HEPATITIS B VIRUS

AND

THE PUBLIC HEALTH

Selected Papers of Baruch S. Blumberg

INDIAN ACADEMY OF SCIENCES
BANGALORE
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**HEPATITIS B VIRUS**


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Acknowledgements

Professor Baruch S. Blumberg visited India during 1986 as Raman Professor of the Indian Academy of Sciences. The Council of the Academy thought it appropriate to bring out a volume of his selected papers in view of the continuing public health interest in the Hepatitis B virus. Prof. Blumberg readily agreed to this suggestion, and we are most grateful to him for having selected this collection himself.

The Academy is indebted to Prof. H. Sharat Chandra, Indian Institute of Science, Bangalore and Prof. S. Ramaseshan, Raman Research Institute, Bangalore for taking the initiative in the planning and processing of this volume. Our special thanks are due to Dr Thomas London, Fox Chase Cancer Center, Philadelphia for contributing an introduction to this publication.

On behalf of the Academy I would also like to thank Dr K.V.S. Rao, International Centre for Genetic Engineering and Biotechnology, New Delhi for the cover photograph. Our sincere thanks are also due to the editorial staff of the Academy.

G. Srinivasan
Editor of Publications
Indian Academy of Sciences
Bangalore.
Professor Baruch S. Blumberg
Introduction

Baruch Blumberg received the Nobel Prize in Medicine in 1976 for discovery of the hepatitis B virus (HBV) and for studies of variation in host response to the virus. At the time of the award it was not appreciated that Blumberg and his colleague, Irving Millman Ph.D., had also invented the first vaccine for hepatitis B, a vaccine that was unique in concept in that it was derived from viral proteins circulating in the peripheral blood of humans chronically infected with the virus. Nor was it realized by the Nobel committee that HBV was the likely cause of most of the cases of primary liver cancer in the world. Nevertheless, the medical faculty of the Karolinska Institute clearly understood that Blumberg had employed methods of human population genetics, methods that were not in use in virology, to discover a virus that had remained hidden to the investigations of a generation of microbiologists. The following biographical information is intended to provide the reader with some sense of the man who was prepared and able to make these discoveries.

Baruch Blumberg was born in New York City in 1925. He attended Union College in Schenectady, New York where he received a Bachelor of Science degree in Physics in 1946. After a year of graduate training in mathematics at Columbia University, Blumberg entered Columbia University’s College of Physicians and Surgeons and received a Medical Degree in 1951. It was rare at that time for physicians to be so strongly prepared in quantitative science. The usual undergraduate majors for physicians in training in the United States during the early 1950s were chemistry or biology.

Medical school introduced Blumberg to the world of research. Between his third and fourth years, he performed public health surveys in an isolated mining town in northern Surinam, South America. This small locale contained an extraordinarily heterogeneous population including American Indians, Hindus (from India), Javanese, Africans, and European Jews. Blumberg observed that these groups responded differently to infection with the parasite *Wuchereria bancroftia*. His report of these observations became his first published paper.

Following medical school, Blumberg did two years of clinical training at Bellevue Hospital in New York City, a public hospital famous for the quality of care provided under incredibly adverse conditions. This was succeeded by two years of study of rheumatology at Columbia University, an experience that fostered further interest in research. Blumberg and his mentor, Charles Ragan M.D., described the natural history of rheumatoid spondylitis, a paper that is still considered the standard in this field. Study of rheumatic disease led to research on hyaluronic acid, an important component of connective tissue. This interest, in turn, led Blumberg to Professor Alexander Ogston’s laboratory in the Department of Biochemistry at Oxford University, where he spent two years as graduate student at Balliol College and received a D.Phil degree in 1957.

Ogston had a powerful influence on Blumberg’s approach to science, even though it did not result in a career in biochemistry. Ogston brought a discipline based on model building and hypothesis testing that might not have been there otherwise. Certainly medical research at most centers in the United States, at that time, was not so formally structured. Ogston and Blumberg have maintained close personal and scientific ties until today.
Another essential component of Blumberg's future research dates from his Oxford days. While there he met Anthony C. Allison M.B., D.Phil. who introduced Blumberg to the concept of genetic polymorphism. It was Allison who showed that carriers of the Sickle Cell hemoglobin trait (heterozygotes) were significantly protected from falciparum malaria. This observation provided the fundamental tenet to Blumberg's future work that genetic variation was responsible for the differences in response of humans to infectious and other environmental agents. Beginning while both were at Oxford and continuing for several years after they left for other institutions, Blumberg and Allison collaborated on studies of polymorphisms of urine, serum and red blood cell proteins in African, Eskimo and American Indian populations. Together they conceived the fundamental notion that humans who were transfused would be exposed to protein variants different from their own and that such exposures would induce the production of antibodies which could be used to detect the polymorphic proteins. Testing of this hypothesis resulted in the discovery of genetic polymorphisms of serum low density lipoproteins. Further testing of this hypothesis, by Blumberg, resulted in the discovery of Australia antigen, which ultimately proved to be the coat protein of the hepatitis B virus.

After Blumberg received his Oxford degree, he accepted an appointment in the National Institute of Arthritis and Metabolic Diseases at the National Institutes of Health in Bethesda, Maryland, where he remained from 1957 to 1964. It was during this period that studies of serum protein polymorphisms were actively pursued and Australia antigen was first observed as a precipitin band in a gel immunodiffusion plate (Ouchterlony procedure).

In 1964 Blumberg moved to the Institute for Cancer Research (now part of the Fox Chase Cancer Center) in Philadelphia. There he continued to study genetic variants of serum proteins, but the main thrust of his research was focused on understanding the nature of Australia antigen. The research team that he organized and directed, including Alton Sutnick M.D., Betty Jane Gerstley M.D., Irving Millman Ph.D. and W. Thomas London M.D., soon identified the relationship of Australia antigen to hepatitis and chronic liver disease and described the variety of responses to what proved to be the hepatitis B virus. These characterizations, although important, could have been done by many research groups. The special stamp that Blumberg gave to this work was derived from his studies of polymorphisms. That gave the research a population and observational focus, as opposed to an experimental or clinical approach. Epidemiology, of course is population based, but the methods that Blumberg used were more like modern genetic epidemiology and molecular epidemiology than epidemiology as it was practiced at that time. It was because of his views on the diversity of human populations that so much effort was devoted to describing the distribution and prevalence of hepatitis B carriers throughout the world. This resulted in an almost immediate appreciation of the global nature of the hepatitis B problem and the need for international solutions. It is not an accident that there have been International Symposia on Viral Hepatitis and Liver Disease every three years since 1972. It is also part of the Blumberg legacy that thousands of investigators throughout the world are involved in some aspect of hepatitis B research.

After a rewarding 25 year career in Philadelphia, Blumberg returned to Oxford in 1988 as the Master of Balliol College. It seems an appropriate completion of the circle. The late Jack Schultz Ph.D., a wise Drosophila geneticist at the Institute for Cancer Research, said that transmission of knowledge had a genetic characteristic in that it was passed vertically from a teacher to his students like traits passed from parents to their children. The faculty and students of Balliol are now benefiting from the "Blumbergian" view of science and the world.

W. Thomas London,
Fox Chase Cancer Center,
Philadelphia, Pennsylvania
GANDHI MEMORIAL LECTURE

Solving and Creating Problems: Hepatitis B Virus and the Public Health

By BARUCH S. BLUMBERG

Raman Professor, Indian Academy of Sciences, Bangalore

Introduction of the Speaker

Today we have with us Prof. Baruch S. Blumberg to deliver the Gandhi Memorial Lecture. He has come to India at the invitation of the Indian Academy of Sciences as the Raman Visiting Professor. He will be spending some time with us, visiting various laboratories, lecturing at different centres and interacting with many young scientists. Most of you would know that he was awarded the Nobel Prize for Physiology or Medicine for the year 1976. The citation says “For discoveries concerning new mechanisms for the origin and dissemination of infectious diseases”.

His career has been an unconventional one for a scientist because after his High School he joined the Navy. His interest in the seas has persisted to this day. In his autobiographical note he reveals its influence on how he tackles his scientific problems. “Sea experience places a great emphasis in problem solving. It involves extensive planning before action and on the arrangement of alternate methods if one is to effect a specific end”.

As a medical student he spent several months in Surinam. While he delivered babies and performed numerous clinical chores he noted the enormous variation in the response of different races to the infection of filariasis. His search for an explanation is the saga of his scientific career. This led him to the Spanish Basques, the Alaskan Eskimos, the nomad Fulanis of Nigeria and the Indians of Quebec. He even spent some time in Chingleput in Tamil Nadu. In this process he became a medical anthropologist of repute.

It is not my intention to deal in detail with his many remarkable discoveries, his work on filariasis, malaria, intestinal parasites, rheumatoid spondylitis; and his discovery of the virus that causes jaundice or the development by him and his colleagues of the vaccine against Hepatitis B infection.

Dr. Blumberg speculates on the nature of scientific enquiry and considers science as a generator and also solver of scientific problems — every time a question is answered several more are raised resulting in an endless search.

I shall end this introduction with two quotations — both often used but particularly applicable to Prof. Blumberg.

“Caring for man and his destiny must always constitute the principal interest of all technical efforts”. – Albert Einstein.

“Recall the face of the poorest and the most helpless man you have seen and ask yourself if the step you contemplate is going to be of any use to him. Will he gain anything by it, will it restore him to a control over his own life and destiny?” – Mohandas Karamchand Gandhi.

May I now request Prof. Blumberg — sailor, traveller, biochemist, medical scientist par excellence and above all a man who cares for the welfare of humanity to deliver the Gandhi Memorial Lecture.

— S. Ramaseshan
Professor Blumberg delivering the Gandhi Memorial Lecture
INTRODUCTION

Professor Sivaraj Ramaseshan, Professor V. Radhakrishnan, Professor Sharat Chandra, colleagues, ladies and gentlemen:

I am honoured to have been invited, in my capacity as Raman Visiting Professor of the Indian Academy of Sciences, to deliver the Gandhi Memorial Lecture for 1986. I have been told that the Raman Trust, which sponsors this lecture, requires that it be held each year, often on the anniversary of the death of Mahatma Gandhi, and within the precincts of the Raman Research Institute.

It is very appropriate that the names of two outstanding Indians, Gandhi and Raman, should be linked. Gandhi was a nationalist in the sense that he recognized the immediate and special problems of his own country and adopted original political and practical solutions to these problems. But he also had the vision to recognize that his methods and his goals had a universal appeal. They inspired many outside India and gave to the world of the oppressed the often-successful political approach of non-violence and the principle of truth-force Satyagraha. In particular, he inspired our own Martin Luther King, whose great contributions have been further recognized this year by the establishment of a United States national holiday to honor his achievements.

Professor Raman also was dedicated to his nation and his homeplace. He maintained his Indian identifications in dress and style even when among his fellow scientists of the West. But he also had the vision to recognize his contributions to world science, which have become even more apparent in recent years. He established the Raman Research Institute and served as the Director of the Indian Institute of Science, institutions of the highest intellectual standards; they have set a demanding standard for the young people of India and offer a powerful means of interaction with scientists in other parts of the world. They can provide the means by which natural observations of phenomena peculiar to India can enter the corpus of scientific problems of interest to the world scientific community.

Before proceeding to the main text of my presentation, I would like to show a photograph (figure 1) which provides an interesting link between Professor Raman and my own institution, the Institute for Cancer Research, Fox Chase Cancer Center, in Philadelphia. It is a picture of Professor Raman and other distinguished x-ray crystallographers, including the late Dr A. Lindo Patterson, much of whose career was spent at the Institute for Cancer Research in Philadelphia. It was taken in 1948 at the First Congress of the International Union of Crystallography at Harvard University, Cambridge, Massachusetts. It pictures Professor C. V. Raman along with J. D. Bernal (England), C. Patacke (U.S.), P. P. Ewald (Germany and U.S.), and the afore-
Figure 1. Professor C. V. Raman with Dr A. L. Patterson (Institute for Cancer Research, Fox Chase Cancer Center) and other scientists at the First Congress of the International Union of Crystallography, Cambridge, Massachusetts, 1948. From left to right: J. D. Bernal, C. V. Raman, C. Palache, P. P. Ewald, A. L. Patterson.
In my talk today I plan to cover several topics.*

1) A description of the research on hepatitis B virus [HBV] and how it can contribute to the public health in India and elsewhere. I will also try to show how broad the research has become over the 20 years it has been in progress and will cite findings of biological interest which have developed along the pathways of research.

2) A review of aspects of scientific process in an attempt to show the problem solving and problem creating character of the scientific method.

3) A discussion of how the solution of an important medical problem concerning HBV generated another problem which then had to be approached by additional attempts at problem solving.

The discovery of hepatitis B virus: Medical applications.

The research which led to the discovery of the hepatitis B virus began as an investigation of inherited variation in humans, specifically by the examination of inherited proteins in the blood. One of the most impressive features of medicine is the great variation in response of this host to disease-causing agents. We reasoned that if we initially studied such serum variation in normal individuals, we could eventually determine how people differ in respect to disease susceptibility and this in turn could help in prevention and therapy. Several of the blood proteins were known to be polymorphic, hence patients requiring multiple transfusions are likely to receive in their blood serum proteins that they themselves had not inherited or acquired. We hypothesized that if some of these proteins were antigenic, a transfused patient might develop serum antibodies against them and his or her blood could be used as a "reagent" to study an inherited serum protein system.

While testing this hypothesis, we identified, in 1961, inherited antigenic variation of the serum low density lipoprotein. Subsequently, we and others found that the sera from selected transfused patients defined an elaborate multiloci system with many alleles controlling serum lipoprotein specificities. This has been used in genetic and forensic studies and may be of value in an understanding of inherited differences in susceptibility to cardiovascular diseases. Since our hypothesis had been successful in revealing a hitherto unknown polymorphic system of variants, we continued to test the serum of transfused patients against a panel of normal sera with the expectation that we would find additional antigen-antibody systems of interest.

The frequencies of genes determining polymorphisms are known to vary greatly from population to population. Hence, to increase the probability of finding unknown phenotypes, we tested the transfused serum not only against sera from the United States (where our work was being done), but also from Europe, Africa, Asia and Oceania.

In one of these studies we detected a precipitin band different from any we had seen before. It developed between the serum of a transfused hemophilia patient from New York and the serum of an Australian Aborigine (figure 2). Our problem now consisted of determining the nature of this unknown material (called "Australia antigen," after the population in whom it was first found, and abbreviated as Au), we tested a large number of sera and found that Australia antigen was rare in normal Americans and north Europeans but common in populations from several areas of the tropics. It was also common in people with leukemia and

* A few references to cited work will be given in this review. A more complete bibliography can be found in references 1 and 2.
this generated an additional hypothesis. Since patients with Down’s syndrome (DS, trisomy of chromosome 21 associated with mental retardation) have a greater risk for leukemia than control groups, we predicted that they too would have a high frequency of Au. We tested sera from Down’s patients and found that about one third of institutionalized patients had Australia antigen.

Studies were made of DS patients with and without Au in order to determine the factors which led to this “condition”. On one occasion we observed that a young man who did not have Au initially, developed detectable amounts of the antigen over a period of weeks. We soon discovered that the appearance of the Australia antigen was associated with laboratory evidence of the development of chronic, asymptomatic hepatitis. This gave us an important clue as to the nature of Au and generated the hypothesis that Au was associated with viral hepatitis.

We tested this hypothesis in turn (in 1967) by simply testing the serum of hepatitis patients, both acute and chronic, for the presence of Au. We soon established that there was a significant association between Au and hepatitis. Having supported this hypothesis, we then created another, namely that Au was on, or was part of, the hepatitis virus. By 1968 we had reported the visualization in the electron microscope of particles associated with the putative virus (figure 3). A series of other observations supported the hypothesis that Australia antigen was part of what soon became designated as hepatitis B virus (HBV)
Even before we had concluded the testing of the virus hypothesis, it became clear that the test we had developed for Australia antigen had a very important application. For many years, a serious and sometimes deadly complication of surgery was the development of hepatitis in patients who had been transfused with blood from a donor who was an occult carrier of hepatitis virus. The donors themselves could be asymptomatic; but if their blood were transfused to a patient, the recipient might develop clinical hepatitis.

In medical research when a finding derived from a basic scientific observation can be used for therapy or disease prevention, an obligation arises to utilize the discovery. By 1969, we advocated donor blood testing and were doing this for the Philadelphia General Hospital. (This was the first hospital in the United States in which donor testing was performed. It illustrates a feature of medical research: the institution in which research is done is often the first place where the beneficial findings are applied.) The “Au test” was also valuable for the diagnosis of acute and chronic hepatitis and became widely used for these purposes.

It appeared then that a medical problem, that is, the prevention of post-transfusion hepatitis due to hepatitis B, had been, to a large extent, solved (particularly when a sensitive radioimmunoassay for HBV was later introduced by ourselves and others). However, the solution had raised other problems, namely the personal, psychological and sociological complications connected with the identification of the carriers. This point will be discussed in greater detail later, after the discussion of other aspects of the hepatitis B research.

Introduction of the vaccine

In 1969, we had received advice from the U.S. Government, the sponsor of much of our research, that we should seek additional funding by finding applications for our basic research results. By that time we realized that antibodies against the surface antigen of the HBV (termed HBsAg) were probably protective. We had rarely seen individuals who had both HBsAg (indicating infection) and the antibody, anti-HBs, in their blood. Further, Okochi in Tokyo had shown that patients who had anti-HBs before they were transfused with donor blood containing HBV were much less likely to develop hepatitis than those who did not have anti-HBs.

Further, we realized that it might be possible to produce a vaccine from the peripheral blood of carriers of the virus. They carried enormous numbers of particles which appeared to be composed only of HBsAg. We devised a method of separating these particles from the whole virus (which was not identified until later by Dane, Almeida and others in England) by centrifugation, column separation, proteolytic enzyme treatment and other methods. The resultant material is treated with formalin and other agents to inactivate any remaining hepatitis virus or any other virus; appropriate adjuvants are added and this constitutes the vaccine.

In 1969, the Institute for Cancer Research filed a patent for the vaccine and its method of manufacture in the United States and foreign countries and it was issued by the United States patent office in 1971. At the time, this was a unique method for producing a vaccine. However, the medical and scientific world was not quite ready for a vaccine since the virus had been discovered only recently, and the validity of the concept was not universally accepted.

By 1975, sufficient amount of work had been done in laboratories in the United States and elsewhere to encourage us to recommend production of the vaccine by an experienced manufacturing company. A licensing agreement was concluded with Merck and Company, a large U.S. drug firm (which had agreed to work on the project as early as 1971) and later others. The vaccine was produced in quantity and in due course, subjected to intensive field testing in the United States. Volunteers from among male homosexuals in New York City were the subjects for the initial trial. It was learned that the vaccine, which
requires three injections over a six-month period, was highly effective (greater than 90% protection) and, within the limits of the trials that had been evaluated, without any side effects. Several million doses of this vaccine have now been used and, to date, there have been no reports of detrimental effects; it is a very safe and effective vaccine. In this case, a vaccine whose major use has been and will be in developing countries, was initially tested on United States populations.

Solution of the vaccine problem raises other problems

The introduction of the vaccine provided an example of how the solution of one problem raised others; in this case, spurious.

In 1980, about the time the use of hepatitis vaccine was beginning to become widespread, the first cases of Acquired Immune Deficiency Syndrome (AIDS) were reported in the United States. Within a few years it became apparent that a terrible epidemic had developed in the susceptible groups, primarily male homosexuals and drug abusers. A small number of cases were also reported in haemophilia patients who had received human blood products, and more rarely in people who had received transfusions or had sexual contacts with people in the high susceptibility groups.

An inordinate fear of human blood products developed; this was extended to the hepatitis vaccine despite the fact that the methods of manufacture destroy all known viruses and that there was no evidence that AIDS had been transmitted by the vaccine. The wide use of the vaccine in the United States (and to a lesser extent elsewhere) was delayed. Subsequently, the agent which is thought to be responsible for AIDS was identified. It was then shown in a variety of ways that AIDS, even if present in the blood from which vaccine is prepared, could not survive the process of manufacture. A further point was the finding that male homosexuals who had received vaccine did not have a different incidence of AIDS than those who had not received the vaccine. Based on these findings, the short-lived fear of the vaccine diminished and it has not impaired its use in areas of the world with high frequencies of hepatitis carriers.

The etiologic role of HBV in the causation of primary hepatocellular carcinoma

There is now a substantial body of evidence that persistent infection with HBV is required for the development of primary hepatocellular carcinoma (PHC). The evidence is sufficiently impressive to have generated large regional and national vaccination programs based on the inference that HBV "causes" PHC. This, therefore, represents the first example of what appears to be a direct, widely used preventive program for a virus-caused cancer and is, in effect, a "cancer vaccine". It is important to stress that the vaccine is not a treatment for cancer that has already commenced.

In the 1950's investigators in Africa and elsewhere had, on the basis of clinical and pathological observations, suggested that hepatitis might precede PHC and be related to its etiology. It was not until the late 1960's when methods for detecting HBV were introduced that it became possible to test this hypothesis directly.

In 1971, at a meeting on cancer in Africa held in Uganda, several studies showing a striking association between HBV and PHC were presented. This event appeared to be a turning point, at least in our laboratory, in the development of interest in this relationship, and the intensity of the work in the field increased. In 1975, we wrote:

"During recent years, there have been parallel developments in understanding, on the one hand, the pathogenesis of primary hepatic carcinoma... and, on the other, the biology of Australia antigen... and the infectious agent, hepatitis B virus (HBV), to which it is intimately related. Recently, the paths of these developments have begun to converge and from this it is possible to design a preliminary strategy which could, if the interpretations of these..."
data are correct, result in the prevention of
many, and perhaps most, cases of one of the
most widespread and deadly cancers of
humans”.

The evidence that was then available and
that supported the statements was presented.
We discussed the vaccine we had introduced
and the possible prevention strategies. In the
relatively short time since that article
appeared, a large body of data supporting the
hypothesis that persistent infection with HBV
is required for the development of most cases
of PHC has been amassed. This data will now
be briefly reviewed [for references see (2)].

Carriers of HBV are common in those parts
of the world where PHC is also common,
including such surprising locations as Alaska
where HBV and PHC are found in high
frequency in the native American population.
A large series of case-control studies has been
completed in which the frequency of the virus
in patients with PHC has been compared with
the frequency in controls. In essentially all of
these, the frequency of persistent HBV infec¬
tion is higher in the PHC patients than in
controls. In studies, which utilized the most
sensitive methods for the detection of the
virus, the frequency of infection among the
patients approaches 100%.

The virus can be identified by immuno¬
histologic methods in the liver tissue of people with
PHC. An interesting feature of this observa¬
tion, shown by Popper and others who have
pioneered in the pathological work, is that the
virus is most abundant in the cells that do not
appear to have undergone malignant trans¬
formation. The neoplastic cells themselves
often have sparse or no evidence of viral
protein, while the apparently non-cancerous
cells surrounding them may have large
amounts of virus.

Currently, one of the most exciting areas of
research is the molecular biology of HBV. The
entire base sequence of the virus has been
determined and confirmed by several groups.
HBV DNA is integrated into the DNA of the
host liver cells in a very large percentage of
PHC cases—in some studies, all the cases.
However, there is also integration of HBV
DNA in the liver cells of patients without
PHC, including patients with chronic liver
disease and asymptomatic carriers of HBV
who have been infected for several years.
Hence, integration itself cannot explain the
pathogenesis of the cancer. The site of integra¬
tion of HBV DNA appears to be the same in
every cell in an individual tumour (i.e. integra¬
tion is clonal), but the points of insertion of
viral DNA are not the same in different
tumours. Based on present knowledge, there
does not appear to be a simple pattern of
integration.

In one of the most convincing studies on
cancer etiology, Beasley and coworkers13 in
Taiwan studied prospectively 22,707 male gov¬
ernment workers between the ages of 40 and 59
years. Recruitment into the study population
began in November 1975 and was concluded in
June 1978. Of the total, 3435 men were
asymptomatic carriers of HBV and the remain¬
der were not. As of December 31, 1983, 116
cases of PHC had developed in the study
population and 113 of these were in HBV
carriers. The annual incidence for the whole
population was 82.4/100,000, but for the car¬
rrier group it was 527.7/100,000. Beasley and
Hwang estimated the relative risk for the
carrier men to be 217 times that of the
noncarriers. The relative risk showed little
variation from year to year and there was no
secular trend. This is probably the highest odds
ratio for any known environmental cause for a
common cancer. On the basis of life table
projections, they estimated the lifetime risk of
death from PHC for a Chinese male carrier in
Taiwan to be about 40%, a remarkably high
figure.

Beasley’s study established that the associa¬
tion between HBV and PHC was closer than
that for any other virus and a site-specific
cancer, probably for any environmental factor
and a specific cancer. It has been shown also
that chronic infection with the virus, rather
than cirrhosis, carries the risk for cancer.
Patients with cirrhosis due to causes other than HBV have a low risk for PHC, while those with cirrhosis due to HBV have a much higher risk.14

Prospective studies similar to Beasley’s are now in progress with Eskimos in Alaska, Chinese in Hong Kong and Singapore, and Japanese in Tokyo. Early reports confirm Beasley’s findings i.e. a greatly increased risk of PHC in the carriers.

In 1971, we had proposed, on the basis of unusual population distribution and clinical and physical characteristics of HBV, that it represented the first identified member of a new group of infectious agents. We termed these “Icrons,” an acronym on the name of the Institute for Cancer Research—where the research on HBV was initiated. Several viruses in this “class” have now been identified in woodchucks, ducks and other species.

The finding of the woodchuck hepatitis virus (WHV) provided another convincing piece of evidence for the hypothesis that HBV and similar viruses cause cancer of the liver. Woodchucks (Marmota monax) (figure 4) persistently infected with WHV have a very high probability (80% or greater) of developing PHC. Uninfected animals or transiently infected animals never or rarely develop tumours. The duck hepatitis B virus (DHBV) found Chinese ducks and in the Pekin breed of domestic ducks (figure 5) is similar to HBV and WHV and it is also associated with primary cancer of the liver, at least in China. Recently, a fourth member of this class of virus which appears to cause primary hepatocellular carcinoma has been identified. Ground squirrel hepatitis virus (GSHV) chronically infects the California ground squirrel (Spermophilus beecheyi) and, based on long-term observations, appears to play an etiologic role in this species. These viruses will be discussed in greater detail later. This appears to be one of the most remarkable examples of a series of viruses causing similar cancers in four different species and provides substantial support to the hypothesis that HBV is a necessary cause of most (or many) cases of PHC.

It has been shown both directly and indirectly that many individuals who develop PHC are infected at a very young age. In some parts of the world, particularly in Asia, the transmission may be maternal. In other locations, transmission is more likely to be horizontal. It is also known that infection early in life...
increases the probability of persistence. These observations favour early childhood vaccination and this will be discussed later.

The hepatitis problem in India

Before proceeding to a discussion of mass prevention of hepatitis B infection, it would be appropriate to discuss the general problem of hepatitis in India.

There are several viral agents which cause the common forms of viral hepatitis. Hepatitis A is caused by an enteric RNA virus quite different in shape and structure from HBV. It can cause acute but rarely chronic hepatitis and is often spread in epidemics by water or food contaminated by the faeces of individuals infected with hepatitis A. There is no vaccine for hepatitis A, but the administration of human gamma globulin can provide protection for about 3 months following the injection.

Hepatitis B can cause both acute and chronic hepatitis, as already noted, and many people may become asymptomatic carriers for long periods of their lives. HBV is spread by many means, by transfer of blood from an infected individual to another, sexually, from mother to child, intimate contact as in families, and probably by insects. It may also be spread by the fecal-oral route i.e. contaminated food and water, but this is not generally thought to be an important mechanism of epidemic diffusion; this question is still unresolved.

There are also other viruses which affect the liver which have not been characterized. These are called “non-A non-B” hepatitis virus since they are neither A nor B; but since they have not yet been identified, a letter designation has not been assigned to them. One of these viruses has characteristics of hepatitis A and can be spread by water and food. Large epidemics of this disease occur in India and in some of these there is a high mortality rate. This is probably the single most common form of viral hepatitis in India.

There is another form of the non-A non-B virus which is similar to B in that it is blood transmitted and chronic. In India this appears to be less common than the non-A non-B virus which is similar to hepatitis A.

Probably the most important single public health measure which could be taken to decrease hepatitis in India would be the control of human waste and the protection of drinking water and food from fecal contamination. Since fecal contamination is the cause of a great deal of disease in addition to hepatitis in India, its control would probably be the most effective method available to help achieve the goal of “Health for all” in India by the year 2000. This is, of course, well known to medical and public health authorities, but the immense logistic effort required has prevented its complete implementation.

I have learned from colleagues in India that, in urban areas, the problem may have increased in recent years because of the rapid growth of city populations. The infrastructure for water supply and piped sewage disposal were in many cases installed years ago and are now inadequate for the increased population. Multiple breaks may exist in the water and sewage lines and, if they are proximate to each other, contamination can occur. This is particularly true when the water supply is inadequate and the pipes are empty for a portion or all of a day. A partial vacuum can result and sewage can enter the water lines if they too are ruptured.

In rural areas, deep bores may provide uncontaminated water, but its distribution after it reaches the surface may allow fecal contamination if proper facilities for control of waste are not used. A major regional and national program directed to providing adequate waste management and clean water and food would result in enormous rewards in health improvement. One approach to this could include the upgrading of training in public health engineering and the improvement in the status of the profession. This might be achieved by attaching schools for training in these disciplines to the high status scientific and engineering institutions in India, such as
the Indian Institute of Science and the Indian Institutes of Technology.

Prevention of hepatitis infection

It has been estimated that HBV is probably the second most common cause of viral hepatitis (after A-like non-A non-B), and it is probably the most common cause of chronic liver disease, including the deadly primary hepatocellular carcinoma. There are estimated to be about 25 million carriers of HBV in India making it, after China (with about 100 million carriers), the second largest concentration of this chronic infection in the world.

Hepatitis B infection from medical procedures

There are several specific strategies that can be used for the prevention, and possibly the eventual elimination of HBV. Prior to the discovery of the methods for detection of HBV in blood, post-transfusion hepatitis (PTH) was extremely common in the United States. In some patients receiving large numbers of transfusions (such as heart surgery), the incidence of PTH events reached 50%. Beginning in the 1970’s, testing of donor blood for HBV was required by regulation and/or law in many United States jurisdictions and in other countries. PTH due to HBV has now essentially disappeared, although PTH due to other viruses (non-A non-B) still occur.

A law enacted by the Government of India in 1976 requires the testing of all donor blood, but for a variety of reasons this has not been accomplished; the Indian Council for Medical Research estimates that only 30% of donor bloods are tested. There are relatively few systematic studies, but the frequency of carriers varies from about 4% in some volunteer blood donor groups to over 20% in professional donors. It has been difficult for me to obtain a figure for the number of blood donor units used in India; 10 million per year has been one estimate. If we assume that an average patient receives two units of blood, then there will be about 5 million recipients of these 10 million units. If we assume a 5% frequency of carriers among the donors (probably a low figure since many donors are professionals), then about 10% of the 5 million recipients will receive at least one unit of blood containing HBV. Based on a prospective study, Dr Jacob John of Vellore has estimated that about one third of patients receiving positive blood from donors will develop clinical hepatitis. Hence, it can be estimated that about 165,000 patients per year will develop hepatitis B from the blood transfusion system.

Another mode of hepatitis infection is the re-use of venipuncture or injection needles or other equipment exposed to human blood. It is difficult to remove encrusted blood from a needle and ordinary sterilization will not kill HBV. The amount of virus which can cause hepatitis in susceptible hosts may be extremely small; an amount contained in the dregs of the needle or other instruments (i.e. blood syringes) could be sufficient.

It is difficult to estimate how many cases can be caused in this manner, but it is probably not less than that caused by transfusions. The preventive measures for these cases are obvious. All donor bloods require testing for HBV using the sensitive enzyme assay (ELISA), radioimmunoassay (less convenient), or a method of equivalent sensitivity. At present there is inadequate indigenous production of the reagents for these assays and the imported reagents are too expensive for widespread use. The encouragement of local manufacture could remedy the problem.

Facilities for the manufacture of sharp disposable needles at low cost would decrease the dependence on re-used needles and instruments. Local manufacture of other disposable blood collecting equipment (i.e. syringes, plastic containers for the units of blood) will also be of value in preventing this mode of infection. It is obvious that the collection of blood from donor groups with a low frequency of HBV carriers (i.e. volunteer donors) would alleviate the situation and probably decrease infection with non-A non-B and possibly other viruses as well.
Prevention of infection with the virus which causes AIDS

There are remarkable similarities between the modes of transmission of HBV and the virus which is thought to cause Acquired Immune Deficiency Syndrome (AIDS). This virus is now called Human Immuno-deficiency Virus (HIV) but was formerly called LAV/HTLV-III. Methods similar to those used for preventing blood borne transmission of HBV can be used for preventing blood borne transmission of HIV. A serological test of donors for HIV (and antibodies against it) can be performed on the same serum specimen required for testing for HBV. Much of the same equipment can be used for the two tests. The techniques described above to prevent transmission of HBV by needles and other equipment will also be effective for preventing transmission of HIV.

There is great demand in India for the prevention of blood borne AIDS. The implementation of both the HIV and HBV programs can be done together, thus effecting large savings in public health expenditures.

Vaccination against hepatitis B

The major preventive programme involves the use of the hepatitis B vaccine. Several strategies have been used in different countries and regions. In areas of low frequency (i.e. United States, northern Europe), routine use of the vaccine has been recommended for high-risk groups such as health care workers, families of carriers, newborn children of carrier mothers, male homosexuals and others. In areas of moderate frequency (say 1–2%, i.e. Japan), the major program is directed to the offspring of carrier mothers. They are given gamma globulin at the time of birth and a second dose of gamma globulin, and the first of three doses of hepatitis B vaccine are given at two to three months of age. A second dose of vaccine is given one month and the third dose three months after the first. In some cases a fourth dose of vaccine may be used. This program has been in progress in Tokyo for several years; it is to be extended to all of Japan within a few months.

In areas of high frequency (such as China), the plan is to vaccinate all newborn children in the high-risk areas. In People’s Republic of China this would amount to more than 20,000,000 courses of vaccinations per year! To accomplish this large goal, indigenous production of vaccine from human blood is being undertaken in newly built factories.

A vaccination program has been in progress on the island of Taiwan since 1984. Taiwan has one of the highest frequencies of carriers in the world; the mean frequency of carriers (among pregnant women) is 18% with a range of 14–22%. Testing was made available to all pregnant women and some 78% were tested (352,721 tested of 450,585 pregnant women). Women who were HBeAg(+) or who had high titers of HBsAg were identified and they were given high titer anti-HBs gamma globulin (HBIG) within 24 hr of delivery; vaccination injections were given at 1, 5 and 9 weeks and at 12 months. A very high percentage (84%) completed three doses and a smaller percentage (71%) the entire course. In July 1986 all newborns will be included in the vaccination program. (Pregnant women will still be tested to ascertain highly infectious mothers and their children will be offered HBIG as before.) In due course, vaccination will be extended to household members of carriers, to medical personnel and eventually to all other susceptibles. National and regional programs are also in progress in the Gambia, Italy, South Korea, United States and elsewhere.

Vaccination program for India

What is the appropriate vaccination strategy for India? A vaccination program could proceed as follows.

1) A systematic survey of Indian populations in different regions of the country to determine the prevalence of HBsAg and anti-
HBs carriers. This would provide knowledge of high infection areas and also an estimate of the population still at risk for HBV infection (i.e. those who have no serological signs of infection).

2) A systematic survey, in different regions of India, to determine the frequency of HBV infection in patients with various kinds of liver disease. This survey could be combined with studies of hepatitis A and non-A non-B hepatitis and would provide some estimate of the disease load of HBV in hospitalized patients.

3) Based on these surveys, strategies for vaccination (possibly different for different parts of the country) could be designed. A decision could then be made concerning target populations for different regions i.e. (i) all newborn children; (ii) only the children of carrier mothers; (iii) susceptibles in the families of carriers and (iv) all susceptibles. Strategies could then be designed, based on the cost of vaccine, disease load imposed by the virus, cost of identifying susceptibles and other variables.

4) The survey and vaccination activities for HBV could be combined with disease surveys and prevention programs caused by other infectious agents. Since there is currently an interest in the prevalence of AIDS virus in India, both surveys could be combined. Eventually, hepatitis B vaccination could be made part of the routine pediatric vaccinations.

5) The cost of vaccine sold in the United States and Europe is very high (about 100 U.S. dollars for the course). However, the cost of vaccine produced in Korea is about one third of this and the Chinese are said to be producing vaccine at about one to three dollars per course. Several studies have now shown that intradermal injections are effective and require only one tenth the amount of vaccine required for the intramuscular route (the method currently widely in use). This brings the cost down to about 10 to 30 cents (equivalent to one rupee 30 paise to three rupees 90 paise), a reasonable amount even for large vaccination programs.

Vaccine prepared by recombinant DNA methods has recently been approved by the Food and Drug Administration of the United States. The cost, however, is not less than the currently available product (that is about 100 U.S. dollars) and it may be some years before methods of production bring it down to the costs of the blood-produced product.

Public health authorities will have to balance the pros and cons of the possible strategies of vaccine production and application and arrive at a judicious decision as to which methods to use.

The biology of hepatitis B virus: Sex differences in response to HBV

Physicians and microbiologists usually encounter viruses and microorganisms primarily in their disease-causing phase. But micro-organisms have a rich interaction with humans and other organisms beyond their ability to cause distress and woe. Examples of how HBV interacts with human sex will now be presented. (For more detailed information and references see refs. 15, 16.)

In many of its responses, HBV interacts differently with human males and females. One of the earliest observations on “Australia antigen” (that is, hepatitis B surface antigen) was the higher frequency of HBV carriers in males compared to females. It was shown that, when infected, males are more likely to become carriers and females are more likely to develop antibody to the surface antigen (anti-HBs). This has an important bearing on the fate of these individuals, since carriers are, in general, more likely to develop chronic liver disease and primary cancer of the liver and those who develop anti-HBs are often immune to further disease. Hence, females are favoured over males in this regard.

Another curious observation emerged from a study of patients receiving treatment on a renal dialysis unit. Prior to the use of sensitive methods for the detection of HBsAg, HBV infection was extremely common in renal
dialysis patients and in some artificial kidney units nearly all patients became infected. (This situation is now controlled and hepatitis infection in dialysis units in the United States and elsewhere is rare. This was achieved by epidemiological control and restricting carrier patients to their own machines in special dialysis units or sections of units.) Many patients on dialysis units were transplanted with kidneys from cadavers and living donors. London and his colleagues found that patients who developed anti-HBs rejected the transplanted kidneys more rapidly than transplanted patients who were carriers of HBV. Furthermore, they found that this effect, although independent of the sex of the recipient, was seen only when the kidney donor was a male. There appeared to be a specific relation between anti-HBs and an antigen present in male but not female kidneys.

The most perplexing observation and possibly the most important, concerns the effect of the response of parents to HBV infection on the sex of their offspring. The original observations were made in a community in northern Greece selected because of the high frequency of HBV carriers. If a mother or father were carriers, the sex ratio of their offspring (number of male offspring divided by the number of female offspring multiplied by 100) was greater than in families in which one of the parents (in particular, the mother) was anti-HBs. Similar observations were made in four other communities; in France, in Kar Kar, Papua New Guinea, in northern Luzon, in the Philippines and in two villages in eastern Greenland. In several of these the increased sex ratio in the carrier families was a consequence of a decreased number of females. There was also an overall decreased fertility in the carrier families.

We have developed several models in an attempt to explain these sex effects. It was suggested that HBV viruses contained an antigen that was present in males but not in females. Hence, when males were infected they would be more likely to regard the antigen as "self," would not develop anti-HBs and hence would be more likely to develop the carrier state. Male kidneys would contain a HBV-like antigen and would be more likely to be rejected by a kidney recipient with anti-HBs. Another model was that the virus was more likely to replicate in a female than in a male fetus and therefore, the female conceptus, infected by the carrier mother or father, was less likely to come to term than the male conceptus. This would explain the observed decrease in the number of girls (and children overall) in the carrier families.

**HBV and insects**

There are other biological interactions of HBV with other species in the environment. The virus is borne by mosquitoes and the field infection rate can be as high as 1:200. It is also found in over 50% of bedbugs (Cimex sp.) (figure 6) captured in beds whose main occupants are carriers of HBV. The virus has also been reported in house flies and other insects.

There is no evidence that the virus replicates in insects; nevertheless, they may be an important source of transmission since, in susceptible individuals only a small amount of virus may cause infection. Insect-borne diseases can be controlled and this may have a bearing on the planning of extended public health programs. This may be particularly important if there is a campaign to totally eliminate the virus; this may be feasible even now in circumscribed island or isolated populations.

**Figure 6. Bedbug (Cimex sp.) SEM.**
Insect infection may also be of biological and evolutionary interest. Insects can provide a mechanism by which genetic information can be carried from one human to another via the viral genome. Since HBV integrates into the host DNA of long-term carriers, the infectious virus may carry with it to an infected person small portions of the genome of the previous host, and this could, theoretically, be expressed in the new human host. An interesting kind of epidemiology may emerge from this in which one is concerned with the infectious agent, the host and the immediately prior host.

Hepatitis B-like viruses in other species

One of the most convincing pieces of evidence that HBV is a necessary cause of primary hepatocellular carcinoma in humans is the existence of a series of viruses, very similar to HBV, which appear to cause PHC in other species. In our earlier investigations, we had postulated the existence of viruses similar to HBV, which we termed icrons.

Four of these viruses have been identified at the Fox Chase Cancer Center by several investigators. These include the human virus (HBV), the virus found in the north American woodchuck or groundhog (Marmota monax) called woodchuck hepatitis virus (WHV), the duck hepatitis B virus (DHBV) first identified in China and the tree squirrel hepatitis B virus (THBV). In addition, a virus has been reported in the California ground squirrel (Spermophilus beecheyi) (GSHV) and recently in the Palm Tree squirrel of India. (The last species is hallowed in Indian tradition. It is the squirrel which assisted Rama when he crossed the sea to Sri Lanka to rescue his consort Sita from the demon Ravana.) HBV-like viruses have also been reported in kangaroos and possibly other species. Primary cancer of the liver, caused by the virus, has been reported in four species (humans, woodchucks, ducks, ground squirrels). These animals are a rich resource for observational and experimental research. In addition, they provide a fascinating body of information to study the evolution of the virus and the diseases they cause in both closely related and distant species.

Solving and creating problems in clinical research

I would like to conclude this presentation with a brief discussion of how, in clinical research, the solution of problems often creates other problems. A brief discussion of certain aspects of scientific process which relate to this concept will be followed by an example from our own experience in research on HBV.

Comments on scientific process in clinical research

A hypothesis is a declarative statement concerning the scientist’s view of a state of nature confined to a specified subject matter. In the inductive phase of scientific process, the data are collected first and the hypothesis induced from them. In the deductive phase, the hypothesis is stated first and then data are collected to test it. After the hypothesis is stated, a study is designed to test the hypothesis (usually an attempt is made to reject the hypothesis) and during the course of testing, a body of data accumulates. Irrespective of the support or rejection of the hypothesis, the data can be used to generate other hypotheses. These, in turn, can be tested by additional data. Again, a decision can be made as to whether the hypotheses of the second cycle are supported or rejected and the data used to formulate a third series of hypotheses, which are also available for testing. This process may be continued through many cycles. As the process proceeds, more and more hypotheses are tested and support or rejection is determined; that is, more and more questions are answered. At the same time, an even greater number of new questions are asked. The more that is known, the greater are the number of unknowns. If perfect knowledge means that the investigator knows everything about a
subject, then, if this model of scientific process
is valid, solutions will always be imperfect. The
solution of one problem will often raise others.

To illustrate this concept, we will use the
experience which resulted from the introduction
of testing of donor blood to prevent post-transfusion hepatitis.

Problem solving and problem creation: The
prevention of post-transfusion hepatitis

The test for what was then known as the
Australia antigen, was reported in 1964. By
1966, as described above, there was substantial
evidence that Australia antigen was on the
surface of hepatitis B virus. In 1967, screening
of blood donors for occult carriers of hepatitis
virus was routine in some hospitals in Philadelphi¬
a and by 1970 it was widely applied in
the United States and other countries.

In 1968, disturbing reports were heard from
persons, usually health care personnel, who
had been identified as carriers of HBV as a
result of a blood donor or hospital staff testing
program. For example, a hospital nurse identi¬
fied as a carrier was told that she would be
dismissed because of the possibility that she
might infect others by personal contact. An
applicant for a hospital position was deemed
ineligible for employment because he was a
carrier. A homosexual man was informed that
he was a carrier but given no specific instruc¬
tion on how he should conduct himself socially.

Along with many incidents of this nature,
policy questions about hepatitis B virus carriers
were raised by institutions. Military medical
authorities in a foreign country where hepatitis
B virus carriers were common asked if they
should screen applicants for admission to
medical school and disqualify those found to be
carriers. The same question was raised with
respect to admission to officers’ training school
and graduation from nursing school: should
these applicants and graduates be screened for
the carrier state to determine if they should be
allowed to enter or undertake the practice of
their chosen professions? (We recommended
that screening should not be done.) Still
another difficult policy problem arose in relation
to the adoption of Southeast Asian refug¬
ee children. In the mid 1970’s, many children
from Indochina were placed for adoption.
Because hepatitis B virus carriers were known
to be prevalent in Vietnam, the advisability of
screening these children for the virus became
an issue. Should the results of this screening
test determine who would be accepted for
adoption and who would not? (United States
health officials decided that carrier testing
should not be done as a qualification for
immigration to the USA from Southeast Asia.)
These carrier-related questions surfaced in
increasing numbers as the use of the hepatitis B
virus test became more widespread.

What appeared to be emerging was the
possibility that a new class of stigmatized
persons and groups—hepatitis B virus car-
riers—was being created by the introduction of
a single laboratory test. These were persons
who did not have any recognizable external
characteristics. Their carrier state was
“occult,” only discernible by use of a blood
test. Many carriers already had been and even
more would be, identified as a result of the
donor and other blood-testing programs that
had been launched. (Tens of millions of donor
blood samples are collected each year, and, in
due course, most of these would be tested.)

It was known that some carriers could
transmit hepatitis readily by blood transfusion.
From this knowledge, but without quantitative
data on actual transmission, it was inferred that
carriers could also convey hepatitis through
social interaction. This assumption began to
take hold despite the fact that it was apparent
that most carriers were not very infectious. It
had been estimated that there were about
700,000 carriers throughout the United States.
If they were infectious—for example, to the
degree that people infected with smallpox
are—there would have been far more hepatitis
in this country and elsewhere than there is
known to be. Furthermore, several preliminary
studies of health care workers known to be
carriers showed that they had not transmitted
hepatitis B virus to their patients. The scientific evidence strongly suggested that, although some carriers might be infectious, many were much less so if at all, and that the danger to public health was probably not immediate or enormous. Nevertheless, persons who had been identified as hepatitis B virus carriers were being medically and socially marked in potentially disadvantageous ways. They were having personal, family and career difficulties as a result of their disclosed carrier status.

Consideration of these problems made it clear that an evaluation of a general screening program should be done before any such program was executed. (This program refers to the screening of persons other than blood donors. Blood donor screening had been evaluated, found to be justified and accepted.) What was called for was a judicious, well-informed set of decisions about screening that would potentiate its public health benefits and protect individual hepatitis carriers from undue economic, psychic and social harm. It became apparent that sufficient data were not available to make sound decisions that would appropriately balance collective needs and individual rights. It was not at all clear what the rules for a screening program ought to be. Should screening be compulsory? Should the activities of carriers be distinguished from those who were not infectious? What instructions should be given to identified carriers? What kind of protection could and should be offered to those with whom carriers came in contact? The history of previous medical applications strongly suggested that these issues should be addressed before the screening procedure acquired the routine familiarity and authority of an established practice; once a procedure has been instituted it becomes increasingly hard to question or freshly evaluate it.

There were also broader issues that had been raised and required consideration. Most of the infectious diseases that people contact are transmitted either directly or indirectly from other people, in many cases from carriers. However, screening for these agents is difficult and not done routinely on large segments of the general population. For example, *Staphylococcus aureus* carried on skin surfaces may be spread from person to person and has caused large and calamitous epidemics in hospital nurseries. Nevertheless, routine screening is not done for this bacteria except after infection has been found. *Salmonella* species may be spread by food handlers and cause serious epidemics of diarrheal disease, but routine screening is not done because of its expense and difficulty. Should hepatitis carriers be targeted for screening simply because the test is easy to do and widely available? Beyond this, how much should biological knowledge be allowed to influence and control our social relationships? To what extent should medical and public health practices be allowed to affect our social behaviour—particularly in the face of the kind of inadequate information that existed at this point about hepatitis B virus carriers and the infectious risk they constitute?

For various reasons, hepatitis screening for blood donors was accepted quickly, whereas screening for many other infectious agents has not been accepted. There was an unambiguous, long-standing need to screen blood donors for hepatitis. Post-transfusion hepatitis was a real and significant problem that had been recognized for years and any solution was bound to be accepted quickly. The tests for hepatitis B virus (particularly the radioimmunoassay that was introduced relatively early in the program) were sensitive and specific. There was a large commercial interest in these tests. Test reagents for hepatitis B virus to the value of tens of millions of dollars are sold yearly, aided by extensive advertising and skilled promotion. Further, several law suits had been brought against hospitals, blood banks, and physicians by defendants who developed post-transfusion hepatitis and claimed that the institution and health care workers were liable because they had not used the screening test for hepatitis B virus. In addition, blood has a powerful symbolic meaning in our culture. It is associated with life and
vigor, lineage and kinship in ways that are likely to confer special positive significance on technical procedures that guarantee its “purity.”

The question of a general screening program (the testing of persons who were not blood donors) had to be viewed very differently. We, and others in the field, took the position that there had not yet been enough research on hepatitis B virus carriers to justify general screening programs, except for those that were part of a research protocol. It was obvious that additional research was necessary to resolve these medical and bioethical problems.

**Advances in research and their impact on ethical questions**

Since our original publications on these issues, there have been scientific and technical advances that have changed the ethical issues in hepatitis B virus carrier screening and altered our views in the process. In 1972, Magnius and Espmark reported their finding of the hepatitis B e antigen (HBeAg). This antigen appears to be a part of the core of hepatitis B virus and its presence in a carrier indicates that significant amounts of whole infectious virus are present in the blood. Mothers who are hepatitis B surface antigen (HBsAg) and HBeAg positive can transmit hepatitis to their offspring, particularly in Asian populations. Carriers with HBeAg are in general more likely to transmit the virus than those without. Furthermore, HBsAg carriers who also have antibody to hepatitis B e antigen (anti-HBe) in their blood are much less likely to transmit the agent than those with HBeAg or those without any sign of HBe antigen or anybody. By separating the carrier group into those who are potentially infectious and those unlikely to transmit hepatitis, the problems of screening were narrowed and focused.

The introduction of the vaccine against hepatitis B virus by Blumberg and Millman (described above) has also markedly changed the medical and ethical picture. If the vaccine continues to be as safe and effective as it now appears to be, it will be possible to protect persons with whom carriers come in contact. In time, in some regions of the world where hepatitis prevalence is high, all or nearly all of the population will either have natural protection against hepatitis B virus (they will have developed the antibody to hepatitis B surface antigen [anti-HBs] after natural infection with the virus), or they will have been vaccinated. When this happens, the public health impact of the carriers will be greatly minimized in these areas. As a consequence, the chief ethical problem of the carrier will have been eliminated.

In areas with low intensity of hepatitis B virus infection, the vaccine will probably be used only in high-risk populations, including health care personnel, travellers, military personnel, blood handlers, homosexual men, drug abusers, family members of carriers, immigrants from HBV high frequency areas and certain other groups. In some of these high-exposure populations, the frequency of naturally occurring anti-HBs may be common (15 to 50%). Because the cost of vaccine is high, it would be prudent in some areas to screen these populations for the presence of anti-HBs (and possibly HBsAg), because they would not profit from vaccination. Under these changed circumstances, the screening of general populations would be warranted. The initial concerns about general population surveys would be set aside, because measures of known value could be taken as a consequence of the survey.

There are other situations in which screening surveys may be warranted. Current policy encourages the “mainstreaming” of mentally retarded children by placing them in small, home-like settings in the general community and in regular schools with other children. There is a relatively high frequency of carriers among children and young adults with Down’s syndrome, who make up a sizable portion of deinstitutionalized, mentally retarded people. Research should continue to determine the risk that these children may impose on their class-
mates and on the feasibility of vaccine protection.

Other advances that would further alter the ethics of screening are likely to occur. Research is now being directed toward an understanding of how to either eliminate hepatitis B virus from carriers, or to decrease virus replication so that the carrier is not infectious. If such measures become possible, they would probably also decrease the likelihood of the carrier developing chronic liver disease or primary cancer of the liver. Under these conditions, identified carriers could be offered a therapeutic procedure. This would provide a powerful new medical and moral rationale for general hepatitis B virus carrier screening that might further offset the negative personal, social, and cultural side effects associated with it.

CONCLUSION

The research on HBV has led to many interesting and curious findings. These have had practical applications and also are of theoretical interest in biology. The search is continuous since the unravelling of one problem generates even more. We hope that it will continue to provide information which will improve health in India and the world.

Introduction to the Papers

Introduction

It is unusual for a scientist to be given the opportunity to contemplate the body of work he has accomplished over the course of a scientific career and to attempt to identify unifying features that bind the contributions together. There is a temptation to seek a grand design in the work that may not have been present in the planning phase, or the scientist may ascribe to himself a subconscious sense of order that prevailed during the course of the work that even he, at the time, did not perceive. It is probably better, to accept the actuality that science proceeds from one unknown to the next (with the hope that some of these will become ‘knowns’) and that the process often follows a ‘Shandean’ course, that is, in the manner of Tristram Shandy, the hero of Laurence Sterne’s novel of the same name, who peregrinates through life in an apparently unplanned and circuituous manner which nevertheless has a strange purpose.

I am indebted to the Indian Academy of Sciences for extending to me this opportunity to indulge in this unusual exercise.

Tropical diseases and physical biochemistry

My first publications were reports on a series of tropical disease surveys undertaken in the small jungle community of Moengo, in the country of Suriname (then Dutch Guiana) located on the northern coast of South America (1, 2, 3)*. I had gone there during my third year in medical school to work in the community hospital (owned by the Suriname Bauxite Company, as was the entire town and the adjacent aluminium mine) and to conduct public health surveys in and around the village and in remote locations in the interior of what was then a primitive and undeveloped country. In retrospect, I find that the impact these amateur projects had on my subsequent scientific work and medical interests was remarkable. The population surveys which were the major part of the Suriname research eventually led to my interest in epidemiology. The work was done in a tropical and undeveloped country; the major impact of the subsequent work of our laboratory and much of the field work which was central to it has been in such locations. The projects were directed towards an understanding of public health and preventive medicine; much of my subsequent research in its application was directed towards prevention and less so towards therapy. But the major impact of the Suriname experience was to focus attention on human variation (and,

* This work was supported by USPHS grants CA-40737, RR-05895 and CA-06927 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania.

* Numbers in parenthesis refer to publications listed at end of book.
in particular, inherited variation) and to question why some people differ from others in respect of susceptibility to disease. This was the subject of much of my research in the 60s and 70s and has been a guiding concept for our subsequent work on hepatitis B virus (HBV).

Following my graduation from medical school (in 1951), I engaged in training and research in Internal Medicine and the subspeciality of arthritis and joint disease. An important area of concern in the arthritis field at that time was the physical chemistry of the large molecules that make up the ‘ground substance’ of the spaces between cells. Hyaluronic acid, a long-chain polysaccharide, was one of these. This substance was of particular interest in that it was the main viscous constituent of the joint fluid and there was reason to believe that physico-chemical aberrations of this molecule could cause or be related to disease. These studies were done in collaboration with Karl Meyer, an eminent physician-chemist who was a pioneer in the chemistry of ground substance, and with Gerald Oster who was at that time working in the laboratory of the pioneer polymer chemist Marks (4, 5). My major work in this field, however, was with Alexander G. Ogston (my supervisor and mentor at Oxford University) with whom I completed a series of studies on the relation between hyaluronic acid and the protein with which it seemed to be invariably associated. We concluded that the protein was an integral part of the proteoglycan molecule, necessary for its biophysical integrity and for the retention of the non-Newtonian viscosity, which was believed to be important for the physiological functioning of the joint fluid (9, 10, 11).

I also developed a clinical research interest during my residency and fellowship. I collected a large body of clinical data on a syndrome called “ankylosing spondylitis”. This was an impressive involvement of the joints of the spinal vertebrae and the sacroiliac joint of the pelvis. It afflicted mostly young men and had a progressive course that led to fixation of the spine and a very noticeable bending and stiffening of the whole frame. By studying individuals at various stages of their disease, it was possible to make estimates of outcome. Contrary to general belief, their functional capabilities (in terms of maintaining gainful employment) were not markedly altered; nor was there a diminution in their life expectancy (8). (This was the first time I used life tables and survival analysis, a device we returned to years later in our studies on disease and morbidity outcomes related to HBV.)

While preparing this study, I became interested in the life of an Irish physician, Bernard Connor, who apparently was the first person to describe the pathology of this condition. This resulted in a paper describing the life and career of Connor written by my wife and myself (19). We became so involved with him that my eldest son received Connor as one of his given names.

**Polymorphisms and inherited variation in response to disease**

While completing my studies on hyaluronic acid at the Department of Biochemistry at Oxford University I met Anthony C. Allison. His research had been heavily influenced by the concept of genetic polymorphisms, an important biological construct that had been developed by Professor E. B. Ford of the Department of Zoology. Based primarily on his experience with butterflies and moths, Ford defined a polymorphism as ‘the occurrence together in the same habitat of two or more discontinuous forms of a species in such proportions that the rarest of them cannot be maintained by recurrent mutation’. The implication of this is that multiple forms of the species are maintained in the population because of differential selection pressure. Independently of the question of selection, which has been much debated in recent years, this principle directed attention to the extensive biochemical and immunologic diversity present in the human genome and provided a grand biological concept which could be used to study variation that might be related to human disease.

At about this time, another development occurred that opened up the possibility of major advances for the study of human polymorphisms. Oliver Smithies, who had worked in Ogston’s laboratory just prior to my arrival, introduced the method of electrophoresis in starch gel. This allowed a much more detailed separation of serum proteins than had been previously possible, and several inherited polymorphisms were detected by combined biochemical, family and population studies. For the next few years, in collaboration with Allison, Michael Tombs (then at Oxford) and others, I studied polymorphisms in human and animal blood in many parts of the world in an effort to discover new polymorphisms and to describe the distribution of those that
were known. Since the polymorphic frequencies were thought to depend on different selective forces operating in the environment, we studied populations living under very different environmental conditions. Thus in 1957 I studied the Yoruba tribe of western Nigeria and the nomadic Fulani of the north (17, 44, 46, 49). The following year, studies were conducted in Alaska among the coastal and inland Eskimos and the Indians of the interior (28, 29, 30, 32, 49). We uncovered a very rich variation, and the differences in the frequencies of the polymorphic alleles we had predicted were easily demonstrated.

These field trips also served to introduce me to anthropologists. Often, on arrival at the remote locations where the blood collections were made the only other outsiders I would encounter were anthropologists studying the ethnology or physical characteristics of the populations. Also, our studies of the populations allowed us to characterize the populations by the gene frequencies of a large number of polymorphic traits: red blood cell groups (12, 28, 30, 46, 49, 79), serum proteins (17, 30, 44, 75, 80, 81), gamma globulin types (49, 75), serum alkaline phosphatase (91, 98), red cell enzymes (62), hemoglobins (61), white blood cell antigens, taste of certain chemicals (32), amino acid excretion in the urine (29, 31, 50), and others. Using the gene frequency characteristics, we could compare populations to each other and conjecture about their historical and genetic relations using existing knowledge derived from history, language and other disciplines seemingly remote from biochemistry. This frequent and intense involvement in field work gave us a more profound understanding of our discoveries than would have been possible if we had merely received a container of blood in our laboratory.

These studies were primarily exercises in data collection, amassing the units of information, which, at some time in the future, could be incorporated into broad concepts. This inductive form of science, in which data is collected from which hypotheses will subsequently be generated, is often disparaged in contemporary science with its emphasis on hypothesis testing and finding answers to specific questions. In many experience, both conceptual approaches are necessary. I enjoyed the opportunity to collect our own ‘raw’ data rather than depend entirely on that of others; it made our work seem more allied to that of the naturalists of the eighteenth and nineteenth centuries who ventured out to new lands to find species of animals and plants from the unknown world to enrich the store of scientific knowledge. We studied many fascinating populations. These included the Indians and Dijkas of Suriname (1, 2, 3), the Spanish Basques (12, 17), Nigerian Yorubas and Fulani (17, 44, 46), Alaskan Eskimos and Indians (28, 29, 30, 32, 49, 98), Micronesians from Rongelap and other atolls of the Marshall Islands in the Central Pacific (31, 38, 39, 43, 44, 50), the Indians of northern Quebec (79, 99) and Peru (130), and others.

**ANIMAL POLYMORPHISMS**

During these field trips we often collected blood from animals other than humans and used these to study the same or similar polymorphisms that we were studying in humans (16, 20, 33, 35, 55). This arose from our interest in the evolution of proteins and the possible connection between proteins known to be inherited in humans and in lower animals. This later developed into the research on hepatitis B-like viruses in species other than man, the phenomenon of the transmission of viruses, and hence genetic information, between animals (particularly domestic species) and humans through the intermediary of insect transmission, and the implication of the latter for the development of massive disease epidemics over the course of history.

Our first paper in this series (16) began with the statement, ‘Opportunity arose during recent trips to Iceland and Nigeria to collect samples of milk from the indigenous herds of cattle.’ We confirmed the presence of a beta-lactoglobulin polymorphism in the Nigerian and Icelandic cattle (Tombs had collected the milk in Iceland and I in Nigeria, both in 1957). We also observed in the starch gel electrophoretic patterns a polymorphic distribution of the alpha-lactalbumins which had not been previously observed, but it was seen only in the Nigerian breeds. The cattle from Nigeria were of the ‘Fulani’ breed associated with the nomadic herdsmen of the same name, whose blood groups and other characteristics we had also studied (44, 46, 49). We conjectured that the relations between Fulani and other cattle might be used to trace the migrations of the domesticated cattle as well as of herdsmen.

On the same expedition to Nigeria, I collected blood samples from several breeds of cattle,
and in collaboration with A. D. Bangham of Cambridge, we studied their hemoglobin polymorphisms (20). This again was used to conjecture about the ancient affinities and migrations of the cattle. Most cattle have only hemoglobin A; however, Jersey cattle have a high frequency of hemoglobin B as do the humped Fulani cattle of Nigeria, and several breeds in southern and central France and in Switzerland. However, B is not found in breeds of northern Europe, nor in the coastal shorthorn cattle of Nigeria (Muturu, N’Dama). We suggested that the B phenotype was associated with Indian cattle from the Indus Valley (Harappan) civilization that had found their way, possibly along with nomadic herdsmen, into Europe and West and East Africa, carrying the hemoglobin B gene with them.

**The Albumin Polymorphism**

In 1966 Liisa Melartin (Prehn) discovered an electrophoretic variant of serum albumin in a population of Algonkian-speaking Indians from northern Quebec, Canada (92). This variant, termed albumin Naskapi after the population in which it was discovered, was inherited and had a very curious distribution (109, 113, 124, 288). In the initial studies it was found only in northern North American Indians (Algonkians, to a lesser extent Plains Indians and northern Athabascans) and some of their southwestern relatives (Apaches, Navajos). Later another variant, albumin Mexico, was identified, and this was found only in southwestern USA and in Mexican and some Central American Indians (104, 303), but neither of the variants was found in European or African populations (277). These variants therefore appeared to be exclusive to American Indians (277). It was not present in Eskimos or Aleuts.

At an unusual meeting dedicated to the origins of Native Americans held in a castle in eastern Austria in 1975, we postulated that, if there were contemporary Asians whose ancestors were remotely related to the Asian predecessors of contemporary North American Indian populations, they might also have the albumin Naskapi variant (299). This prediction was soon fulfilled. Electrophoretic variants similar to albumin Naskapi were identified in certain Turkish (306) and Punjabi (India) populations (332). Recently it has been shown that the Turkish variant has the same amino acid substitution as the variant from the American Indian population, a finding unlikely to be due to chance (378). We have also found that there are differences in the binding of coumarins (plant-derived drugs), to the different albumin variants (268), and this may be of medical and evolutionary significance. The specific amino acid substitutions in other albumin variants have also been identified (303).

The human albumin polymorphism provides an exciting opportunity not only for the study of migration into the New World, but also for the study of the evolution of human serum proteins.

**Isoantibodies in the Blood of Transfused Patients**

By the year 1960 it became apparent that there was considerable inherited variation in the serum proteins based not only on our own experience but on that of others who by this time had discovered polymorphisms of the haptoglobins, transferrins, gamma globulins, Gc proteins and others. If that were the case then transfused patients would be likely to receive proteins that they had not inherited, given the known frequency of the ‘average’ serum protein polymorphisms. Allison visited my laboratory in Bethesda that summer and we decided to test the hypothesis that some of the known or unknown polymorphic proteins might be antigenic in humans who had not themselves inherited or acquired the specificity. We used the recently introduced Ouchterlony technique of double diffusion in agar gel to test this hypothesis. The serum of the transfused patient was placed in the central well of a seven-well pattern cut in the gel and sera from other individuals were placed in the peripheral wells (see Figure 1, reference 43). If an antibody existed in the serum of the transfused patient, then it would diffuse into the gel and react with the specific antigen if the latter were present in the sera in the peripheral wells.

Our prediction was soon fulfilled; after about fifteen trials we found the serum of a transfused patient (Mr C. deB.) with a refractory anemia that gave a clear and beautiful precipitin reaction with the serum of some but not all individuals against which it was tested. In fact Mr C. deB.'s
serum could be used to divide humans into two distinct groups, those whose blood reacted with his and whose blood did not. Using family studies, we showed that the presence of the ‘reacting’ phenotype was inherited as a simple autosomal dominant (57) trait. We termed this the ‘Ag’ system.

The protein carrying this inherited antigenic variant was soon identified as the low density lipoprotein (54), a protein species associated with heart disease, diabetes, cardiovascular disease and other maladies of considerable importance. We (and others) found additional inherited specificities of the low density lipoprotein (64, 74, 76, 101, 102). Gradually it appeared that there was a complex series of closely linked loci (perhaps four) at each of which two or more alleles were segregating. Much of this complex genetics and immunology was accomplished by a group of European and Japanese scientists (Vierucci, the late Jan Hirschfeld, Butler, Okochi and others), some of whom we collaborated with over the years. One of the problems in the field was the difficulty in establishing standard typing of sera as had been done for the Gm polymorphism, and later, brilliantly, for the white blood cell antigens (HLA groups). The WHO convened a conference in an effort to establish uniformity but this proved unsuccessful. Probably for this reason the Ag system never blossomed and, despite the importance of low density lipoprotein in the genetics of cardiovascular and other diseases, it has been little employed.

**Discovery of Australia antigen**

However, by this time we were on to other matters. We had continued our survey of sera from transfused patients based on the notion that a continued search might reveal additional polymorphic, antigenic proteins. In 1963 Harvey Alter (an investigator from the Blood Bank of the National Institutes of Health, Bethesda, USA, who was working in our laboratory), saw a precipitin band that formed between the serum of a transfused hemophilia patient from New York City and a single serum in the panel of 24 against which it was being tested. It differed considerably from the lipoprotein precipitin bands we had previously seen. This single serum was from an Australian Aborigine (73). The lipoprotein phenotypes we had previously described were common in all the human populations in which they had been tested, but the new protein was rare. Following a practice common in human genetics, we gave it a geographic name, ‘Australia antigen’ (Au), after the place of origin of the blood in which it was discovered (84). Assigning a proper name, a name that did not infer any supposed function or purpose, had a distinct advantage in that it made clear that we did not know what it was and that any interpretation was possible. The problem at this point was to determine what this antigen was (93) and how it figured in the life, and possibly the death, of humans.

We determined that the precipitin reaction was stable even with sera that had been stored frozen for years. Hence we were able to complete extensive studies on the thousands of sera we had collected and stored. We found that Au was rare in the United States and North European populations, but common in African and Asian populations, and generally, in tropical areas (96). We also tested the hypothesis that it was inherited as a simple autosomal recessive trait. This was supported in the early studies (96, 127), but exceptions to the expected segregations were found in subsequent studies. We also showed (127) that inheritance of Au was closely associated with susceptibility to persistent infection by a hepatitis virus. The possibility of inheritance hepatitis virus infection has still not been dismissed, but our increasing knowledge of the genetics of the hepatitis B virus and its host in the 1980s outdistanced these early genetic speculations. We decided to abandon the studies on segregation in families and await the possibility of a direct test on the genetic material itself.

**Australia antigen and the hepatitis B virus**

There had been several observations suggesting that Au might be related to hepatitis but the proximate clue which focused the research on the hepatitis association was the finding that Au, although rare in normal Americans, was common in people who had leukemia (84, 100, 115, 148, 164). We made several hypotheses based on this observation. One was that Au was related to the virus that was supposed to be the cause of leukemia, a second that leukemia led to the presence of Au. Another
hypothesis stated that both leukemia and Au were caused by the common inherited factor. This hypothesis could be tested quickly since there were several groups of people known to be at high risk of developing leukemia. Children born with Down’s syndrome (trisomy of chromosome 21 associated with mental retardation) have a much higher likelihood of developing leukemia than do other children. We predicted that there would be a high frequency of Au in Down’s syndrome patients; a study was completed in a large institution for the mentally retarded in Maryland, and the prediction was confirmed. A second and then a third study were conducted in other institutions in New Jersey and Pennsylvania, and each of these supported the original observations (100).

We conducted a series of observational studies on the Down’s syndrome patients. We had made the initial observation that the presence of Au in the serum was persistent; if present at one testing, it would be present at subsequent testings, and if absent initially, it would be absent later. We observed an exception to this in one of our patients. A young man, Mr James Bair, who had been initially negative on a test, subsequently developed Au. He was admitted to the hospital for observation. Coincident with the development of Au, he had developed a form of anicteric (that is, without jaundice) hepatitis.

This observation, then, generated the hypothesis that Australia antigen was associated with hepatitis. It was tested in a simple-minded way. We showed that the frequency of Au in persons with diagnosis of viral hepatitis, based on the then existing clinical criteria, was higher than in a control group. Our first report was based on only 48 cases of hepatitis and was included in a paper (100) on Australia antigen, Down’s syndrome and leukemia. We subsequently submitted a larger study, but this was rejected by the Annals of Internal Medicine. Years later, the person who had reviewed and rejected that paper told me that he had done so because he believed (correctly) that we were claiming to have identified the hepatitis virus, and since this had supposedly been done so many times in the past, he disbelieved our findings. This has allowed us to indulge in the perverse pleasure of believing that we were struggling against the scientific establishment to gain acceptance of our findings. This was to some extent true, but it did not seem, in the long run, to have greatly retarded the clinical use of our initial findings. It may, however, have had some effect on the introduction of the vaccine (see below).

We separated the rejected report into two parts. In one, the frequency of Au in acute hepatitis was reported (120). In the second, we compared the levels of enzymes (e.g. SGPT or ALT) that are elevated in liver disease in patients with Down’s syndrome with and without Au (108, 181). Both of these supported the hypothesis of association. In a later paper, we also showed an association of Au with several forms of chronic hepatitis (131).

There was an interesting and curious direction that our work took at this time (late 1960s). Au had been found in high frequency in patients with leprosy. This led to an extensive series of studies on this fascinating and tragic disease (103, 139, 141, 152, 156, 215). The data were used in our genetic studies, in the hepatitis-association investigations, and in other applications. The major impact of the leprosy work, however, was to focus our attention on the immunologic aspects of HBV infection. We found that the carrier state for HBV was associated with the lepromatous rather than the tuberculoid form of leprosy. The former is associated with major immunologic abnormalities. It also caused us to consider again that there was an inherited susceptibility to hepatitis B virus and the organisms which cause other diseases, i.e. leprosy. It also raised the conjecture that a potential ‘host’ might have a specific ‘knowledge’ of HBV before infection, a notion that has become more interesting in recent years when homologies for the HBV genome are being sought for and found in the human genome (the so called variable number tandem repeats, or VNTR).

Now that the association of hepatitis with Au had been confirmed (by ourselves and, later, others), we set about to test the hypothesis that Au was, or was a part of, one of the hepatitis viruses. In collaboration with Manfred Bayer, a colleague at the Institute for Cancer Research, we visualized (in the electron microscope) particles associated with the Au fraction of the serum (114). It was later appreciated that these were particles consisting only of surface antigen and could be distinguished from the whole virion particle which was subsequently identified by British investigators. Millman developed a method for immunofluorescent identification of Au in tissues and showed that it was localized to the liver cells in individuals infected with HBV (126, 140). The antigen was also found in other tissues in humans and other primates using this technique. We had already seen that Au could be transmitted to some individuals by transfusion, while others
exposed in the same way would develop an antibody against it; these were characteristics expected of a virus.

It has proven very difficult to grow the virus in standard tissue culture lines, but Coyne and Millman were able to grow the virus in organ cultures from livers of people infected with HBV (180). It is curious that many of the standard methods (tissue culture, plaque formation, standard laboratory animal inoculation, egg inoculation, etc.) for the isolation and identification of viruses were not satisfactory for HBV. In fact the virus had been detected in quite a unique manner, viz., immunodiffusion in agar gel using the serum of a transfused patient. It was perhaps because none of our group had been trained as a virologist that we used these unconventional but, in this case, appropriate methods rather than the standard approaches that proved to be unsatisfactory. In any case, by the early 1970s, the scientific community in general accepted that the hepatitis virus had been identified.

Prior to our work, it had been recognized that there was more than one kind of hepatitis virus. The virus associated with Au was most similar to what had previously been referred to as hepatitis B virus (HBV), which appeared to be transmitted primarily by blood, as in transfusion. The Au-associated virus was transmitted in that manner, but also, obviously, in other ways since the vast majority of people infected with it had never been transfused nor had been injected with a needle. Other modes of transmission — sexual, maternal, possibly by insects, the faecal–oral route — were suggested by epidemiological observations. In the early 1970s it was accepted that Australia antigen was the surface antigen of the hepatitis B virus. The distinction between hepatitis B and other hepatitis viruses (i.e. hepatitis A) was made possible by our findings building on those of Krugman and other early workers in the hepatitis field. Shortly after, very sensitive tests, in particular, radioimmunoassay, were introduced (177). This soon resulted in an immediate application to the testing of blood donors to detect HBV carriers; post-transfusion hepatitis due to HBV has now been virtually eliminated in many countries (356). The events leading to this clinical application have been described (356). In a very short time, our initial laboratory findings were translated into practical medical application.

**Invention of the hepatitis vaccine**

Millman had in 1967 come to work in our laboratory with a nearly unique experience. He had been employed by Merck, a large pharmaceutical company with facilities near Philadelphia, where his major task was to produce a purified pertussis vaccine; he was well prepared for the development of a vaccine against hepatitis B.

We recognized that carriers had in their blood large numbers of particles of a material that was probably the surface antigen of the virus. In our first isolations, we had not been able to detect significant amounts of nucleic acid (147), and in a series of experiments using non-human primates we found that, whereas a partially purified preparation of the Australia antigen particles given by injection appeared to result in transmission of the disease, highly purified preparation did not. We reasoned that the Au particles contained only the surface antigen (HBs) of the hepatitis B virus and that the purification process separated it from the infectious, DNA-containing particles that alone would be responsible for transmission.

We had also observed that Au antigen and the antibody against it (anti-HBs) were rarely found in the same individual, and the Japanese workers (Okochi and others) had shown that transfused patients who developed anti-HBs were significantly less likely to become infected after transfusion with blood containing Au than patients who had not developed anti-HBs. These observations were consistent with the explanation that antibody developed against Au — the surface antigen — was protective.

In 1969 we described a method for making a vaccine from the surface antigen particles present in the blood of carriers and a patent for this process was issued in 1972 (188, 275, 321, 348, 377, 382). Interest in the vaccine was increased as a result of a series of experimental studies with humans and chimpanzees by Krugman, Hilleman, Purcell, Maupas and others. In 1975 we made arrangements with a local pharmaceutical company (Merck), and later with companies in France and other countries, for the manufacture of the vaccine. Extensive field trials (notably by Szmuness) demonstrated the effectiveness and safety of the vaccine, and it is now widely used.
For example, in 1987, the People's Republic of China began a program for the universal vaccination of all new born children; some 25 million babies per year will be included in the program.

The introduction of the vaccine probably represents the major contribution of our laboratory to the improvement of public health.

**Sex differences in response to HBV infection**

There are striking differences between males and females in response to infection with HBV (298). Our first observations showed that there were more carriers among males than females (96, 191). We later demonstrated that males, when infected, were more likely to become carriers than females, and females more likely to develop anti-HBs (203, 273B). In our laboratory and elsewhere, a maternal effect on transmission was shown. Mothers were more likely to transmit the virus to their offspring than fathers (179, 225), and carrier mothers had slightly longer gestation periods than mothers who were not carriers (194).

Perhaps the most surprising finding was a relation between the parents' responses to infection with HBV and the sex of their offspring. Carrier parents were more likely to have boys than girls, and this pattern was seen in very different environments: Greece (251, 289), Greenland (367), Papua New Guinea (320) and the Philippines (323). If these findings are confirmed, they imply that HBV may have a profound effect on one of the most important demographic and evolutionary factors in human biology. Since similar viruses occur in other animals, and since HBV DNA can integrate into host DNA, there are fascinating implications for the role of this virus in the genetic composition and destiny of human populations. The interaction of behavioural and biological effects was dramatically demonstrated in a study in a Melanesian population where young males are traditionally raised by their fathers and uncles, and spend much of their youth in 'men's houses'. As might be expected, in their society father-to-son transmissions were more common than in other cultures (350).

**HBV and the aetiology of primary hepatocellular carcinoma**

At a meeting in Kampala, Uganda, in 1972, reports from various parts of the world clearly showed a higher frequency of infection with HBV in patients with primary hepatocellular carcinoma (PHC) than in control groups. Shortly after this meeting, we decided to direct most of the efforts of our laboratory to testing the hypothesis that persistent infection with HBV was required for the development of PHC (256). This was substantially supported in subsequent studies in laboratories and in field studies in Asia, Africa and elsewhere by a number of investigators. By the early 1980s it was generally accepted that HBV was a necessary causal agent for PHC, one of the most common cancers in the world. This evidence is reviewed in several of our publications (311, 358, 364, 371).

Our earliest studies on the association of HBV and PHC (133) used the insensitive immunodiffusion method and did not show a relation. They did, however, inform us that the levels of the virus are low in individuals with clinical cancer, an important finding in relation to the disease model we later formulated. Much of our field work on PHC was conducted in Senegal and Mali in West Africa and in Korea. The African studies were accomplished with a group of French and African colleagues (Larouze, Payet, Barrois, Saimot, Marinier, Sankale, Fere, Diop, Froment, Sow, Diakhate and others). The Korean studies were conducted by Hann of our staff and collaborators (Kim, Bae) (359).

In Africa (246) and America (309) we showed a strong association of PHC with HBV using a variety of sensitive tests, including using the antibody against the core of the virus, anti-HBc. We demonstrated in Africa (262) and Korea (325) that carriers who went on to develop PHC were more likely to have been infected in their early childhood than carriers who had not developed PHC; the infection was often from their mothers. We also showed in extensive cross-sectional and prospective studies that children in Africa are unlikely to become persistently infected before about one year of age (262), but after that infection is likely. This information was valuable in designing vaccination strategies. In indicated that priority should be given to children under one year of
age. In practice, in the large national programs now in progress, children are vaccinated at about three months of age when other childhood vaccines are administered. Risk factors leading to PHC were analysed in the African studies (271, 294). One of the major risk factors for the development of PHC appears to be elevated ferritin levels (see below).

Studies on the aetiologic relation between HBV and PHC, to which our laboratory contributed, provide an impressive body of data on the viral cause of a common human cancer. It is the only viral-caused cancer for which an effective vaccine is available, and has spurred national programs for direct intervention to prevent PHC. Universal childhood vaccination and/or other vaccination strategies have been initiated in the People's Republic of China, Taiwan, Korea, Gambia, in Italy, Singapore, Hong Kong, Philadelphia and elsewhere (358).

In 1982 we developed a model (322), subsequently elaborated, to explain the relation between HBV and PHC. It proposed that HBV can replicate only in differentiated cells, which then become susceptible to destruction. Cells not fully differentiated when infected do not allow replication and are at a selective advantage. They divide rapidly, in response to the death of the infected differentiated cells, and eventually a mutational event converts them to 'cancer' cells. This model has been very helpful in the design of new concepts and experiments.

**Viruses similar to HBV present in other species**

In 1971 we predicted (169) the existence of viruses similar to HBV that would have a carrier status, a polymorphic distribution, and the propensity to cause cancer as well as other forms of disease. We termed these 'Icrons', an acronym after the Institute for Cancer Research (304).

The first new virus discovery came about through contacts at the Philadelphia Zoo. We learned that there is a high frequency of primary cancer of the liver in *Marmota monax*, the common woodchuck. This resulted in the discovery and characterization of woodchuck hepatitis virus (WHV) by Summers and others at our institution (374). A virus in a related species, the tree squirrel (*Spermophilus beecheyi*), was discovered in California, and a third virus was identified in tree squirrels in our laboratory (368, 370). An HBV-like virus has also been found by Mehrotra in the three-banded palm tree squirrel of India.

While travelling in China in 1977, I was told that in regions of China where PHC is common in humans, it also occurs in certain breeds of domestic ducks. Sera obtained from one such breed eventually resulted in the discovery of duck hepatitis B virus (DHBV), which, in China at least, is associated with liver cancer. Other viruses have been reported in other animal species but have not yet been well characterized.

It is likely that many species of animals will have their own variety of hepatitis B-like viruses. This can provide an interesting evolutionary thread connecting the host animals and the viruses that infect them. It also raises questions of a possible role for these viruses in transmitting portions of human and other genomes (possibly via insect vectors), representing a method for causing major gene frequency changes in populations in which widespread viral infection occurs.

**Insect vectors**

We first identified HBV in mosquitoes in collections in Uganda and Ethiopia (220) in 1972. High field infection rates for several species were then noted in collections in Senegal by Wills and others, and feeding experiments were conducted in collaboration with colleagues in Kenya (221, 259). Wills and Ogston found that bedbugs captured in beds whose main occupants were HBV carriers had a more than 60% frequency of HBV infection. It was also shown that bedbugs retain the virus for several weeks in their bodies and excrete it for an even longer time in their faeces (278). Ogston demonstrated experimentally that the virus could be transmitted from one container to another by bedbug vectors.

The presence of the virus in insects does not necessarily mean that it is transmitted by them; there is, for example, no evidence that the virus replicates in the insects. However, the virus exists in very high concentration in the blood of many carriers, there is an extremely high field infection rate in areas where insect – human contacts are very frequent, and susceptible people may become
infected after exposure to only a very small amount of virus; hence insect transmission remains a possibility.

HBV has many characteristics of a retrovirus, including integration into the genome of infected hosts. The virus replicating in a host may contain some of the genetic information of the host, and these, theoretically, could be transmitted from person to person by an insect vector — a flying, crawling mechanism for the transmission of viral and host genes.

IRON AND IRON-BINDING PROTEINS

There are factors in addition to infection with HBV that are required for the development of PHC and/or that accelerate the carcinogenic processes in an individual chronically infected with HBV. We decided to study iron and iron-binding proteins because there was a significant body of data indicating that these factors have a major impact on infection and cancer (366). Also, the amount of iron in an individual can be manipulated by regulating intake or by removing iron from the body with chelating agents. We reasoned that if a biologically significant relation were found, it might be possible to develop therapeutic strategies for altering iron and iron binding.

We found that iron is increased in HBV carriers (239, 296) and that this appears to be a consequence of infection and does not precede it (315). Our interest in the tissue iron-binding protein ferritin developed as a consequence of the studies our colleague Hann had conducted on the relation of increased ferritin levels to poor prognosis in childhood neuroblastoma. Lustbader found that individuals with elevated ferritin, when exposed to infection with HBV, were more likely to become carriers than individuals who had lower levels, and the latter were more likely to develop anti-HBs (336). The elevation of ferritin preceded the infection.

We then used an epidemiologic approach made possible by the large collections of sera we had stored in our field trips extending back to the 1950s. Sera had been collected in the Solomon Islands by Damon and his colleagues in the 1960s and we had participated in some of the blood studies. The sera had been frozen and stored. In the 1980s, Richard Stevens, then a doctoral student of mine went to the Solomon's and determined who was alive or dead. The sera samples collected earlier were tested to determine the ferritin and transferrin levels. As predicted, the individuals who had died had higher levels of ferritin and lower levels of transferrin, that is, higher body iron stores, than those who had survived. These studies have been called 'historic prospective' in that the specimens are of the past, but they are utilized in a prospective fashion.

A similar technique was used in a cancer study in Taiwan. It was shown that increased iron stones earlier in life increases the probability of certain kinds of cancer later in life. These and similar studies by ourselves and others (primarily Stevens) may lead to a reconsideration of the practice, common in many countries of fortifying foodstuffs, particularly bread, with iron. We have recommended that iron should be prescribed only for those who need it since increased iron intake in the general population might increase total body iron stores and possibly increase the probability of decreased survival and cancer.

In a prospective study in the Solomon Islands, Stevens et al found that an increased ferritin level is a predictor of shorter survival (343), and in a similar study in collaboration with Beasley in Taiwan (369), we found that increased ferritin levels were prognostic of increased probability of cancer. Both these studies utilized our large collection of sera obtained during field trips, and population and clinical studies. We have collected sera for more than 25 years and more have been discarded. In the Solomon Islands study, sera collected in the 1960s and 1970s were used. In the 1980s individuals who had died and those who had survived were identified and their sera examined for levels of ferritin. A similar technique was used for the cancer study in Taiwan. This constitutes a powerful form of 'historic-retrospective' epidemiologic study.

All these findings, in addition to ongoing experimental studies, indicate that increased iron may hasten viral replication and the development of cancer. We are currently designing therapeutic trials to test this notion.
Infection with HBV had been a major problem in renal dialysis and in renal transplantation units. As many as 50% of patients on a unit might become infected; epidemics among the staff were frequent and in some instances with many fatalities. The development of the test for the Australia antigen made it possible to detect carriers and control the epidemics using conventional epidemiologic principles.

In the United States in the 1970s, legislation had been passed which authorized the Federal government to pay for renal dialysis. This allowed many people to obtain treatment, and new dialysis units were opened. We were approached by physicians from a newly opened dialysis unit in the Philadelphia area and asked to help in managing the hepatitis problem. In some locations, we were told, patients who were identified as HBV carriers were denied treatment; if they were not accepted at another unit, this amounted to a decision to let them die. London advised against this procedure, and we agreed to monitor patients and staff and provide advice on appropriate practices. In addition, carriers were identified and assigned to a special unit. Within a short time the regular units were essentially free of HBV. Similar practices have been adopted in other renal dialysis and transplantation centres. Widespread epidemics and endemics with HBV are now rare (273), and this was accomplished by the use of simple principles of epidemiology and public health control.

London, Drew and the others involved with this project made some fascinating observations during the course of the ten-year follow-up program on the dialysis units. They established that males were more likely to be carriers and females to develop anti-HBs after infection (see below). They also found that individuals who developed anti-HBs were more likely to reject transplanted kidneys than patients who, prior to transplantation, had become carriers (266, 279, 283). Further, in some of the studies this difference could be seen only if the transplanted kidney was from a male donor! This suggested that the virus shared antigens with human tissues, and that these were more common in males.

Other studies

Over the years, I have maintained an interest in the history of science and in the process of medical research (307). This has been reflected in several publications, for example, the short biography of Bernard Connor already mentioned (19), and a description of an enormous pathological specimen, namely arthritis in the vertebrae of the dinosaur Diplodocus (45). I also described what, at the time, appeared to be the earliest artistic rendition of a human goitre in a haut-relief from Gandhara, a Greek-influenced culture in what is now the northwestern portion of the Indian subcontinent (77, 97).

There have been several articles on bioethical problems related to the stigmatization of individuals recognized as carriers (e.g. 261). The problems with HBV preceded the very difficult issues now being raised concerning the identification of individuals who carry the AIDS virus, but, for hepatitis, the problems were handled in a totally different manner. I also pointed out how science both solves and creates problems and coined the term 'Daedalus effect' to characterize this phenomenon (360, 377).

We identified multiple specificities of HBV (121) and added to the fascinating story on the relation of these specificities to the anthropology of population movements (150, 204, 214, 224). Before we realized that Australia antigen was a part of the hepatitis virus, there were several cases of hepatitis among our staff. We described this epidemic and then recommended measures for the control of the disease in the laboratory. We introduced the neologism 'ergasteric', that is, disease generated in a laboratory (167), a word that has not gained wide acceptance. Drug abusers were identified as a high-risk group for HBV infection (163). In collaboration with military physicians, we established that many servicemen who had been in Vietnam contracted HBV infection, and that much of this could probably be accounted for by intravenous drug abuse (232).
As our knowledge of chronic HBV infection increased, particularly after the development of the model for the HBV-PHC relation, it became clear that a therapy against HBV might be effective even if it did not completely eliminate HBV. If a drug did not totally eliminate the virus, but only decreased the rate of pathogenesis, it might be possible to keep a person asymptomatic throughout his or her life-time. We referred to this as "prevention by delay" (331). We predicted that such a drug might be found in plants, since so many of the drugs in current use derive from plants. We compiled a list of plants that had been used in indigenous medical systems for the treatment of jaundice and other symptoms of liver disease. We concentrated on plants that had been used in three or more continents as having the greatest promise for further study. Prominent among these were plants of the genus Phyllanthus, a pan-tropical weed. Independently of this selection process, Venkateswaran, in our laboratory, had been studying this plant because of his personal knowledge of its use in India. Extracts of this plant have now been tested extensively in vitro and in vivo and the results have been very promising (374). It is hoped that Phyllanthus may be developed as a drug that could be used on the two hundred million or so HBV carriers in the world to markedly decrease their risk of chronic liver disease and PHC.

Baruch S. Blumberg
HEPATITIS B VIRUS
A “New” Antigen in Leukemia Sera

The “Australia antigen” is found in the sera of some normal individuals from foreign populations. The total absence of the antigen from the sera of normal United States subjects and its relatively high frequency in acute leukemia suggests that the presence of the antigen may be of value in the diagnosis of early acute leukemia. Whether the antigen results from or precedes the leukemia process remains to be seen.

Baruch S. Blumberg, MD, Harvey J. Alter, MD, and Sam Visnich

Patients who receive large numbers of transfusions for anemia and other causes may develop precipitins in their blood. These precipitins may react in agar gel double diffusion experiments with specific human serum lipoprotein found in the blood of other individuals. Since these precipitins were found only in patients who had received transfusions they were thought to be antibodies against serum lipoproteins which developed in the patients as a result of the repeated transfusions. The precipitin is referred to as an isoprecipitin since it develops against a specificity found in an individual from the same species. The antilipoprotein isoprecipitin developed in approximately 30% of 47 patients with thalassemia who had received transfusions. Isoprecipitins also developed in smaller number of transfused patients with other diseases. All precipitins stained with sudan black, a dye specific for lipid. Immunoelectrophoretic and ultracentrifugal studies showed that the protein with which the isoprecipitins reacted was a low density lipoprotein. The reactivity of the isoprecipitin is inherited as an autosomal-dominant trait and several lipoprotein specificities have been found.

In 1963, sera from patients with hemophilia who had received transfusions were tested for the presence of isoprecipitins using a panel of 24 sera from normal individuals, including sera from foreign populations. Two of the hemophilia sera formed a clearly defined precipitin line with one of the panel sera (from an Australian aborigine), but with none of the others. In contrast to the usual findings the precipitin line stained only faintly with sudan black; it did, however, stain with azo carmine, a general protein stain (Fig 1). Subsequent studies have shown that this protein system differs from that detected with the antilipoprotein antisera. The serum protein with which the hemophilia isoprecipitin reacts has not been fully characterized and has been tentatively called the “Australia antigen.” This paper will describe the epidemiologic and immunologic aspects of the Australia antigen-isoprecipitin system.

Materials and Methods

Double diffusion in agar gel was done using a micro-Ouchterlony technique on lantern slides. Each of the two hemophilia sera containing the isoprecipitin was placed in the center well of a seven-hole micro-Ouchterlony pattern. The sera to be tested for presence of Australia antigen were placed in the peripheral wells. When a panel of antigen-containing sera were identified in this manner, they in turn were each placed in the center wells of similar seven-hole Ouchterlony patterns, and the sera of patients who had received transfusions, which were to be tested for the presence of isoprecipitins, were placed in the peripheral wells. In the final testing program two sera-containing Australia antigens which reacted with all the hemophilia antisera first discovered, were selected to screen for the remaining antisera. Two of the strongest hemophilia antisera were used in screening for the sera containing Australia antigen. In screening for antilipoprotein antisera, the sera from patients who had received transfusions were tested using a panel of 24 sera selected from four or more population groups as in previous studies.
Formation of precipitin line between serum from leukemia patient (top well) and hemophilia serum (bottom well).

Immuno-electrophoresis was done by a modification of the method of Grabar and Williams. The precipitin lines were first stained with sudan black and then with azo carmine. Sera were fractionated by ultracentrifugation in high density salt medium. Fractions with a specific gravity greater and less than 1.063 were prepared.

A total of 28 hemophilia sera were studied. These included samples from patients from Mt. Sinai Hospital, NY, New York Hospital, and the Clinical Center of the National Institutes of Health. They were all United States whites and had all received transfusions many times with fresh frozen plasma and whole blood obtained from the hospital blood banks as well as commercial sources. The racial makeup of the donors could not be known with certainty, but they probably included United States whites and Negroes. The hemophilia serum used in the immuno-electrophoresis experiments contained antibodies against both lipoproteins and the Australia antigen. It was obtained from a patient (1) who had received more than 900 transfusions.

The sources of some of the normal sera used in the studies are given in the Tables. The sera from patients were obtained at the Clinical Center, National Institutes of Health, New York Hospital, and elsewhere. The calculation of the probability value was done by using two by two tables and Fisher’s χ-square method.

**Results**

**Frequency of the Isoprecipitin.**—The sera of 107 patients who had received approximately ten or more transfusions, and of 150 normal individuals who had not received transfusions, were tested for the presence of the Australia isoprecipitin (Table 1). Eleven isoprecipitin containing sera were found in the patient group; none was found in the normal population. Of these 11, eight were patients with hemophilia, one had plasma thromboplastin component deficiency disease (PTC), one thalassemia, and one aplastic anemia. The frequency is highest in the hemophilia and related PTC group, and the difference from the other groups combined is statistically significant (*P*<0.001). It had previously been found that the frequency of antilipoprotein isoprecipitin was very high in thalassemia as opposed to other patient groups. Hemophilia sera containing the Australia isoprecipitin were tested for the presence of antibeta lipoprotein; they were found to have an incidence similar to other patients who did not have thalassemia. Only one of the patients in the latter group had an anti-Australia antigen isoprecipitin.

There were two sera which contained both antilipoprotein and anti-Australia antigen isoprecipitins. This is approximately the number expected by chance.

**Frequency of Antigen.**—A total of 1,704 sera from nonhospitalized and presumably normal subjects were tested for the presence of Australia antigen using at least two, and generally more antisera (Table 2). Reactors were found only in Oceanic, Oriental, and Mediterranean populations, and in one from the American Indian population. No reactors were found among the approximately 700 United States sera tested including cord serum from 18 newborns. The highest frequencies were

<table>
<thead>
<tr>
<th>Disease</th>
<th>Antibody to Australia Antigen</th>
<th>Antibody to Lipoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Present</td>
</tr>
<tr>
<td>Hemophilia</td>
<td>28</td>
<td>8</td>
</tr>
<tr>
<td>PTC*</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Thalassemia</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>Other Diseases</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td>11</td>
</tr>
<tr>
<td>Normal controls</td>
<td>150</td>
<td>0</td>
</tr>
</tbody>
</table>

*Plasma thromboplastin component deficiency disease.
found in a small group of native Taiwanese and in Australian aborigines. All of these tests were done on stored sera.

Sera from a total of 659 patients with various illnesses were tested against one or more of the antisera as shown in Table 3. Of the ten reactors found, eight had leukemia (including one patient with a diagnosis of both acute lymphocytic leukemia and multiple myeloma, and one patient with a newly described preleukemia syndrome associated with a missing chromosome in marrow cells\(^{10}\)) and two had thalassemia. These patients with thalassemia were siblings whose parents were born in Greece. As noted in Table 2, the frequency of reactors in presumably normal Greeks is 4\%. The difference between the frequency in leukemia patients and that in all the disease groups combined is statistically significant \((P<0.001)\). All of the patients with the Australia antigen had received transfusions. However, approximately 300 of the patients who did not have the Australia antigen had also received transfusions.

The leukemia sera were studied in greater detail. The antigen was found in all the larger subclasses with the exception of chronic myelogenous leukemia. The patients with reacting sera were of both sexes, and their ages at the time of the diagnoses varied from 6 to 55 years. Of the ten patients in whom the antigen was found, eight are now dead, including all but one of the eight patients with leukemia, and one of the patients with thalassemia.

A total of 54 patients with acute leukemia were studied. Of these, six had the Australia antigen and were all dead. Of the 49 who did not have the Australia antigen, 19 were dead. The sample size is too small to permit adequate corrections for age, duration and intensity of disease, and other factors, but there does appear to be a gross difference with respect to mortality between these two groups.

**Characteristics of Antibody and Antigen.—**The original isoprecipitin-containing sera and the reacting sera had been found using specimens which had been collected and stored at \(-20\ C\) for up to 27 months. The Australia antigen was present in sera as well as in sera or plasmas stored for up to six years. Several specimens collected at different times were subsequently obtained from patients who had received transfusions and whose sera contained the isoprecipitins, and from patients with leukemia and normal subjects whose sera contained the Australia antigen. These studies showed that the isoprecipitin was present in sera or plasmas which had been stored at \(-20\ C\) for up to 27 months. The Australia antigen was present in fresh sera as well as in sera or plasmas stored for up to six years. These studies also indicate that within the limits of observations, the presence of the Australia antigen is essentially an invariant characteristic of the individual; that is, if it is present at one point in time, it is present when tested subsequently.

The isoprecipitin appears to be a 7S \(\gamma\)-globulin.\(^9\) Australia antigen migrates in the beta and slow a-globulin region on immunoelectrophoresis, but can readily be distinguished from the antigen which reacts with the antilipoprotein isoprecipitin found in the serum of patient 2 as shown in Fig 2. (The top basin contains the antilipoprotein antisem from patient 2, and the second basin serum from hemophilia patient 1. The latter has isoprecipitins directed against both the Australia antigen and lipoprotein. The electrophoresis well contains the serum of a patient with leukemia which reacts with serum from patient 2 and with both of the isoprecipitins in serum from patient 1. Two distinct lines of slightly different mobility were seen between the sera of patients 1 and 3. The lower one, which stains blue with sudan black, corresponds to the patient 2 lipoprotein line. The upper one, which stains only faintly or not at all with sudan black, is not a lipoprotein.)

**Table 2.—Australia Antigen in Normal Populations**

<table>
<thead>
<tr>
<th>Population</th>
<th>No. Tested</th>
<th>Australia Antigen Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aborigines, Australia</td>
<td>208</td>
<td>12, 6</td>
</tr>
<tr>
<td>Chinese, USA and Taiwan</td>
<td>65</td>
<td>0, 0</td>
</tr>
<tr>
<td>Eskimo, Alaska(^9)</td>
<td>24</td>
<td>0, 0</td>
</tr>
<tr>
<td>Greeks, Greece(^{10})</td>
<td>179</td>
<td>8, 4</td>
</tr>
<tr>
<td>Indians, Canada(^{16})</td>
<td>78</td>
<td>0, 0</td>
</tr>
<tr>
<td>Indians, Mexico</td>
<td>100</td>
<td>1, 1</td>
</tr>
<tr>
<td>Israelis</td>
<td>96</td>
<td>2, 2</td>
</tr>
<tr>
<td>Japanese, USA</td>
<td>48</td>
<td>0, 0</td>
</tr>
<tr>
<td>Koreans</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Micronesians, Rongelap(^{18})</td>
<td>193</td>
<td>7, 4</td>
</tr>
<tr>
<td>Negro, USA(^{17})</td>
<td>241</td>
<td>0, 0</td>
</tr>
<tr>
<td>Newborn children, white</td>
<td>18</td>
<td>0, 0</td>
</tr>
<tr>
<td>Polynesians, Bora Bora</td>
<td>24</td>
<td>1, 4</td>
</tr>
<tr>
<td>Samaritans, Israel(^{18})</td>
<td>125</td>
<td>2, 2</td>
</tr>
<tr>
<td>Taiwanese</td>
<td>23</td>
<td>3, 13</td>
</tr>
<tr>
<td>Tristan da Cunha Islanders</td>
<td>42</td>
<td>0, 0</td>
</tr>
<tr>
<td>Vietnamese</td>
<td>24</td>
<td>1, 4</td>
</tr>
<tr>
<td>White, USA (NIH(^*) employees)</td>
<td>215</td>
<td>0, 0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1,704</strong></td>
<td><strong>38</strong></td>
</tr>
</tbody>
</table>

\(^*\)National Institutes of Health.

**Table 3.—Australia Antigen in Patients**

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. Tested</th>
<th>Australia Antigen Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abetalipoproteinemia</td>
<td>4</td>
<td>0, 0</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>15</td>
<td>0, 0</td>
</tr>
<tr>
<td>Anemia</td>
<td>18</td>
<td>0, 0</td>
</tr>
<tr>
<td>Arthritis, various(^*)</td>
<td>15</td>
<td>0, 0</td>
</tr>
<tr>
<td>Cancer (other than leukemia)</td>
<td>47</td>
<td>0, 0</td>
</tr>
<tr>
<td>“Connective tissue” disorders†</td>
<td>5</td>
<td>0, 0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>96</td>
<td>0, 0</td>
</tr>
<tr>
<td>Hemophilia</td>
<td>24</td>
<td>0, 0</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>17</td>
<td>0, 0</td>
</tr>
<tr>
<td>Leukemia</td>
<td>70</td>
<td>8(^\dagger), 11.4</td>
</tr>
<tr>
<td>Acute myelogenous</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>Acute lymphocytic</td>
<td>38</td>
<td>2</td>
</tr>
<tr>
<td>Chronic myelogenous</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Chronic lymphocytic</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>45 chromosomes(^{19})</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Lupus erythematosis</td>
<td>69</td>
<td>0</td>
</tr>
<tr>
<td>Multiple myeloma and macroglobulinemia</td>
<td>93</td>
<td>1(^\dagger), 1.1</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>11</td>
<td>0, 0</td>
</tr>
<tr>
<td>Rheumatic fever</td>
<td>124</td>
<td>0, 0</td>
</tr>
<tr>
<td>Tangiers Island disease</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Thalassemia</td>
<td>48</td>
<td>2, 4.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>659</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>

\(^{1}\)Includes eight patients with rheumatoid arthritis, three with psoriatic arthritis, and four with Sjögren’s disease.

\(^{2}\)Other than lupus erythematosis.

\(^{3}\)One patient had both chronic lymphocytic leukemia and multiple myeloma, and is included in both categories.

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does stain with azo carmine.) Using the hemophilia serum from patient 1 (see above), two distinct lines with slightly different mobilities can be seen. One of these stains blue with sudan black and corresponds to the lipoprotein antigen which reacts with patient 2. The other, which has a slightly different mobility and a different curvature, does not stain with sudan black, but does stain with azo carmine. In addition, a reaction of nonidentity can be demonstrated by immunodiffusion when the Australia isoprecipitin system is compared with the typical lipoprotein precipitin as shown in Fig. 3. (The serum of patient 2 contains an antilipoprotein isoprecipitin. The serum of a patient with thalassemia with an isoprecipitin against Australia antigen is in the top right hand well. The serum of the Australian aborigines has both the specific lipoprotein and the Australia antigen. The patient-2 line stains blue with sudan black and the patient-4 line stains red with azo carmine.) Additional characteristics of the Australia antigen and a method for its partial isolation are described in another paper."

Specificity.—To determine if the antisera had different specificities, they were all tested against the same antigen placed in the center well of a seven-hole-Ouchterlony pattern. No crossing of lines were seen between the antisera. Similar experiments were used to compare the Australia antigen found in the presumably normal subjects with that found in the leukemia patients and no differences could be detected. On the basis of these initial experiments, we have not been able to detect any specific differences between the various antisera, nor between the Australia antigen found in normal persons, and in leukemia patients.

Family Studies.—The studies on normal populations were done on sera collected on field trips for other purposes and stored in the Institutes serum bank. In some cases, family sera were available, and the results were analyzed to determine if there was a family aggregation of Australia antigen. Batsheva Bonné examined 125 sera from Samarians living near Tel Aviv, Israel* (Fig 4). This represented nearly all the members of this highly inbred community. Of these, two siblings who were the offspring of a consanguineous marriage (both of whose parents were double cousins) were the only individuals with detectable antigen. In the Micronesian population, there was one father and son affected, but in the five other cases the individuals were only remotely related or unrelated. The reactors in the Greek study were not apparently closely related. Of the 47 patients with thalassemia studied, only two were positive, and they were siblings. Their parents and one aunt were not reactors. The two brothers of one of the patients with leukemia with the Australia antigen did not react with the antisera.

Comment

A second kind of isoprecipitin system has been revealed by this study. The Australia antigen with which the isoprecipitin reacts differs from the lipoprotein antigens previously described and the distribution of isoprecipitins and reactors in patients and in normal populations is very different from that found for the lipoprotein system.

It has not been firmly established that the isoprecipitin in the hemophilia sera is an antibody nor that the protein with which it reacts is an antigen. However, by analogy with the lipoprotein system, it is probable that this is the case and these terms have been used in describing the system.

In our discussions it has been assumed that the "antibody" is present in the sera of patients with hemophilia and other patients, and the "antigen" in the leukemia and normal sera. This appears likely since the patients with hemophilia and other patients had all received transfusions whereas some of the individuals in whose sera the Australia antigen was found had not. The possibility still remains, however, that the rare normal sera and leukemia sera actually contain an antibody against an antigen present in hemophilia patients. It is hoped that further studies with patients with hemophilia may help resolve this point.
The high incidence of this isoprecipitin in the hemophilia-patient group suggests that either hemophilia per se predisposes to the formation of the isoprecipitin or that the administration of fresh frozen plasma (which distinguishes the treatment of hemophilia and PTC patients from the other individuals who had received transfusions) particularly predisposes to the formation of the antibody. Preliminary evidence suggests that the Australia antigenic sites may be revealed by a freezing process which leads to the denaturation of lipoprotein. This possibility will be discussed in another paper.*

The high incidence of the Australia antigen in the leukemia population as compared with normals or other patient populations suggests either that; (1) persons with the Australia antigen have an increased susceptibility to the development of leukemia, or (2) the antigen itself is a manifestation of the disease process, perhaps secondary to an alteration in some normal serum constituent with a resultant change in antigenic configuration, or (3) the Australia antigen is related to the virus which has been suggested as the cause of leukemia.

The first hypothesis implies that the Australia antigen is present in a patient before he develops signs and symptoms of leukemia. The long-term observations necessary to support this possibility have not yet been made. The Micronesians, Samarians, and Australian aboriginal populations would be suitable for such a study. In the case of the "preleukemic"10 (Table 4) patient the presence of the Australia antigen predated the development of frank leukemia.

Changes in red blood cell antigens during the course of leukemia lend indirect support to the hypothesis that the Australia antigen results from, rather than precedes the leukemia process. Many authors have demonstrated alterations in red blood cell ABO specificities during the course of leukemia.11 More recently, it has been shown that there is a loss of I specificity on the red blood cells of certain patients with leukemia.12 The I antigen is almost universally present in normals and patients with other diseases.

The third hypothesis is being tested in collaboration with workers at the National Institutes of Health.

The total absence of the Australia antigen from normal United States subjects studied and its relatively high frequency in acute leukemia suggests that the presence of the antigen may be of value in the diagnosis of early acute leukemia.

The available data are too few to support a genetic hypothesis, but none of the family studies are inconsistent with simple recessive inheritance of the specificity.

4. Portion of a pedigree from the Samaritan community, representative of nearly all members, showing the presence of the Australia antigen and the close interrelation of the parents.
Summary

An isoprecipitin is present in the sera of many patients with hemophilia who have received transfusions. It reacts with a protein (the "Australia antigen") that is found in the sera of some normal individuals from foreign populations but is absent in sera of the United States populations studied. It is found in approximately 10% of patients with leukemia.

R. Rosenfield, MD, and E. Smith, MD, of Mt Sinai Hospital provided the hemophilia antisera. Other valuable sera used in the experiments were provided by T. D. Dublin, MD, Marion Erlandson, MD, J. Fahey, MD, H. H. Fudenberg, MD, R. Kirk, PhD, L. Lauster, MD, L. Rosen, MD, C. Sheba, MD, N. R. Shulman, MD, and A. G. Steinberg, PhD.

Samples of sera on patient 1 were provided by J. M. Hill, MD, of the Wadley Research Institute and Blood Bank, Dallas.

This investigation was supported in part by Public Health Service grants CA-08069-01 and CA-06551-02 from the National Cancer Institute.

References


The Impartial Negative.—So far as I know there are only a few medical journals in the world whose proprietors employ a full-time editor, among them the Journal of the American Medical Association and its counterpart in the United Kingdom, the British Medical Journal, which I have the honour to edit. I use the word "honour" deliberately, though in the context it has a very commonplace and pedestrian ring about it. To edit the British Medical Journal is regarded as an honour, and I have often wondered why. It is a harassing, exacting, worrying, and ungrateful kind of job. Much of one’s time is spent in saying “No!” to writers of monographs, articles, books, and letters, and to advertisers—self-advertisers and the legitimate trade. Each year I have to reject solely on grounds of space some two thirds of the articles submitted for publication in the British Medical Journal. These disappointed authors cannot feel otherwise than a bit cast down, and however polite the formula of rejection they will consider the editor an obstacle in the way of their progress. As a full-time editor who no longer practices medicine or works in the laboratory, I often feel acutely uncomfortable when returning to an author a paper which is the result of weeks, maybe months, of patient observation by the bedside and experiment in the laboratory—work summed up in voluminous tables, histograms, and the like, work, one has sadly to reflect at times, which had much better be left undone or done in a different way. At least, I feel, that is some justification for the existence of full-time medical editors who do not practice their original profession. By looking on and seeing all sides of the game, they do stand a chance of achieving impartiality. They can sum up the opinions of two or three expert referees on a paper, and come to the final decision which every editor has to make: “Yes” or “No.” It is in the exercise of this judicial capacity that a medical editor is taxed most severely. He needs a fund of that rare commodity, common sense; and ability to assess evidence and a keen nose for the humbug, the self-advertiser, for the man who wants to sell something.—Clegg, H.: An Editor’s Prejudices, Int Rec Med, vol 168, Oct, 1955.
WE HAVE PREVIOUSLY REPORTED THE PRESENCE OF AN ISOANTIGEN OF HUMAN SERA, RATE OR ABSENT IN NORMAL U.S. AND NORTHERN EUROPEAN POPULATIONS BUT RELATIVELY COMMON IN PATIENTS WITH LEUKEMIA (1, 2). THE "NEW" ISOANTIGEN HAS BEEN CALLED "AUSTRALIA ANTIGEN" BECAUSE IT WAS FIRST FOUND IN THE SERUM OF AN AUSTRALIAN ABORIGINE. IT APPEARS TO BE DIFFERENT FROM THE LOW-DENSITY LIPOPROTEIN ISOANTIGEN SYSTEM FIRST DESCRIBED IN OUR LABORATORY (3-5). HEMOPHILIA PATIENTS AND OTHERS WHO HAVE RECEIVED MULTIPLE TRANSFUSIONS MAY DEVELOP PRECIPITATING ISOANTIBODIES AGAINST AUSTRALIA ANTIGEN. THESE PRECIPITATING ISOANTIBODIES REACT WITH A PROTEIN IN THE BLOOD OF SOME NORMAL AND SOME SICK INDIVIDUALS. THIS ISOANTIGEN (AUSTRALIA ANTIGEN) COULD NOT BE IDENTIFIED AS BEING ANY OF THE KNOWN SERUM PROTEINS.

AUSTRALIA ANTIGEN (AU(1)) HAS THE IMMUNOELECTROPHORETIC MOBILITY OF AN ALPHA GLOBULIN AND A SPECIFIC GRAVITY OF LESS THAN 1.21 (6). IT STAINS FAINTLY WITH THE LIPID-SPECIFIC DYE SUDAN BLACK, INDICATING THE PRESENCE OF SOME LIPID. IT CAN BE DISTINGUISHED FROM THE SERUM ALPHA- AND BETA-LIPOPROTEINS BY ITS STAINING CHARACTERISTICS, FLOTATION PROPERTIES, AND IMMUNOLOGICAL SPECIFICITIES. SPECIFIC ANTIBODIES AGAINST AUSTRALIA ANTIGEN HAVE BEEN PRODUCED IN RABBITS BY IMMUNIZATION WITH WHOLE SERUM THAT CONTAINS AUSTRALIA ANTIGEN FOLLOWED BY ABSORPTION WITH THE SERA OF INDIVIDUALS WITHOUT THE PROTEIN (7).

RECENT STUDIES OF BLOOD COLLECTED IN CEBU, PERU, AND RONGELAP ATOLL SHOW SIGNIFICANT FAMILY CLUSTERING OF THE TRAIT (8). IN ADDITION, THE SEGREGATION DATA ARE CONSISTENT WITH THE HYPOTHESIS THAT INDIVIDUALS HOMOZYGOS FOR AN ALLELE Au^ (GENOTYPE Au^/Au^) HAVE DETECTABLE AUSTRALIA ANTIGEN (PHENOTYPE Au(1)) WHILE THOSE HOMOZYGOS FOR AN ALTERNATIVE ALLELE (Au/Au) AND HETEROZYGOTES (Au^/Au) HAVE NO DETECTABLE ANTIGEN (PHENOTYPE Au(0)). PENDING THE ACQUISITION OF ADDITIONAL DATA, THIS GENETIC HYPOTHESIS HAS BEEN PROPOSED TO EXPLAIN THE FAMILY CLUSTERING IN THE POPULATIONS TESTED.

WITH THE USE OF HUMAN AND RABBIT ANTISERA, THE DISTRIBUTION OF Au(1) IN SEVERAL NORMAL AND PATIENT POPULATIONS HAS BEEN DETERMINED (2, 8). Au(1) IS ABSENT IN NORMAL U.S. POPULATIONS AND RARE IN NORTH EUROPEAN POPULATIONS (APPROXIMATELY 1/1,000) BUT IS FAIRLY COMMON IN SOME SOUTHEAST ASIAN AND MEDITERRANEAN POPULATIONS. IT IS ALSO RELATIVELY COMMON IN PATIENTS WITH LEUKEMIA, PARTICULARLY IN THOSE WITH ACUTE GRANULOCYTIC LEUKEMIA (SEE BELOW).

THE ORIGINAL FINDING OF Au(1) IN VERY LOW FREQUENCY IN NORMAL INDIVIDUALS AND IN
relatively high frequency in some kinds of leukemia suggested that individuals with this trait (which they may have inherited) may be more susceptible to leukemia than those without it. A corollary of this hypothesis is that individuals with a high risk of developing leukemia would comprise a population having a higher frequency of the trait than subjects drawn from the normal populations. Several such groups are known. These include patients with Down’s syndrome (mongolism), polycythemia vera, co-twins of an identical twin having leukemia, individuals exposed to radiation, and others (for a review, see Miller (11)). Several of these groups have been tested for the presence of Au(I), and the highest frequency in any U.S. population so far tested has been found in a group of patients with Down’s syndrome (9, 10).

In the present paper, we describe our further studies on the prevalence of Au(I) in Down’s syndrome patients.

Material and Methods

Blood specimens were obtained from 75 male residents of the State Colony at New Lisbon, N. J., who were admitted with the diagnosis of mongolism. For a control group, blood specimens were obtained from 76 male residents with the diagnosis of ‘epilepsy.’ The age distribution was approximately the same as in the Down’s syndrome group, and they had been institutionalized for approximately the same length of time. The admitting diagnoses of residents at New Lisbon State Colony are supplied by the patient’s local physician, and these in general are not altered after admission. Chromosome studies were performed on 17 of the Down’s syndrome patients and 4 of the controls (see below).

After the identification of Down’s syndrome patients with and without detectable Australia antigen, a second collection was undertaken in which blood was withdrawn from 75 residents with Au(I) and 26 residents without it. A series of tests were performed on these samples. The white blood cell, red blood cell, and platelet counts, the white blood cell differential count, hemoglobin concentration, and hematocrit volume were determined using standard laboratory methods. We are indebted to Dr. R. A. Donato of Jeanes Hospital, Fox Chase, Philadelphia, Pa., for performing these tests. The following red blood cell antigens were identified using the appropriate antisera: B, A, A, C, D, D, E, C, e, Le, Le, Kp, Kp, M, N, S, s, Vel, K, k, Fy, Fy, Jk, Lu, Lu, Ja, P, Tj, and V. We are indebted to Dr. Neva M. Abelson of the Hospital of the University of Pennsylvania for performing these tests. Uric acid levels were determined by the method of Liddle, Segmiller, and Last (12) through the courtesy of Dr. L. A. Healey of the University of Washington. The white blood cell glucose-6-phosphate dehydrogenase and galactose-1-phosphate uridyl transferase levels were determined by Drs. W. J. Mellman and F. A. Oski of the University of Pennsylvania using the methods described by them (13). The vitamin B levels were determined by the method of Hutner, Bach, and Ross (14). We are indebted to Dr. W. J. Williams of the University of Pennsylvania for performing these tests.

Blood specimens from nine Down’s syndrome residents (five girls and four boys) of a Maryland institution for mentally retarded children were also tested. Blood from patients with polycythemia vera was obtained at three different clinics. These included 14 patients from Philadelphia, 36 from Portland, Ore., and 32 from San Francisco. Other sera from patients were collected primarily in the Philadelphia area, as noted in the Acknowledgments section. Venous blood was collected into dry Vacutainers (Becton, Dickinson Co.) or test tubes. The blood was allowed to clot overnight and the serum, removed by centrifugation. In some cases, small amounts of sera were separated from the blood immediately after collection and used in the double diffusion tests. Sera were stored at —20 C for later testing.

Double diffusion in agar gels was done by a micro-Ouchterlony technique described previously (15). Double diffusion in agar gel tubes was done by the method of Preer and Preer (16). Chromosome studies were performed on leukocyte cultures from 17 of the Down’s syndrome patients using a method recently described (17).

The chi-square determinations were done as described by Snedcor (18). When any of the two-by-two table cells contained numbers lower than five, Fisher’s exact method was used. Student’s t test was used for comparing the quantitative results obtained on the antigen-positive and -negative bloods. Where appropriate, the method for comparing unpaired series of numbers was used.
### Results

**Distribution of Australia Antigen in Patients**

Nearly 30% of the Down’s syndrome patients had detectable Australia antigen in their serum. This includes (Table 1) 19 of the 75 males from the New Jersey institution and 3 of 5 females and 3 of 4 males from the Maryland institution. This represents the highest frequency found in any patient group so far tested. Four of the 76 control patients had Australia antigen. Of these, three had some stigma of Down’s syndrome, that is, prominent epicanthal folds, Brushfield’s spots, large interpupillary distance, short middle phalanx of the fifth finger, “mongoloid crease” of the palm, high palatal arch, etc. However, none of these had the typical Down’s syndrome chromosome pattern on examination of the peripheral blood. As noted below, mosaicism cannot be ruled out by chromosome analysis of a relatively small number of cells from a single source. The difference in frequency of Australia antigen between the Down’s syndrome and the control group is highly significant ($\chi^2 = 11.8$, $P < 0.001$).

The protein was not found in any of the normal individuals from the United States. Australia antigen has been found in patients with leukemia, hemophilia, and thalassemia as well as in those with Down’s syndrome. Forty-eight sera from patients suspected of having serum hepatitis were tested. Of these, five were found to have Australia antigen. The findings in hepatitis will be described in greater detail in a subsequent paper. In

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**Table 1. Distribution of Australia Antigen in Patients and U. S. Normals**

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. Tested</th>
<th>No. Positive</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-beta-lipoproteinemia</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anemia, various</td>
<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arthritis*</td>
<td>70</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cancer, other than leukemia</td>
<td>95</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diabetes†</td>
<td>303</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Down’s syndrome (mongolism)‡</td>
<td>84</td>
<td>25</td>
<td>29.8</td>
</tr>
<tr>
<td>Fanconi’s anemia (hypoplasia bone marrow)</td>
<td>2</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Hemophilia</td>
<td>60</td>
<td>3</td>
<td>5.0</td>
</tr>
<tr>
<td>Hepatitis, virus‡</td>
<td>48</td>
<td>5</td>
<td>10.4</td>
</tr>
<tr>
<td>Hodgkin’s disease†</td>
<td>12</td>
<td>1</td>
<td>8.3</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leukemia and related diseases (see Table 2)¶</td>
<td>177</td>
<td>16</td>
<td>9.0</td>
</tr>
<tr>
<td>Lupus erythematous</td>
<td>69</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Multiple myeloma and macroglobulinemia¶</td>
<td>95</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polycythemia vera</td>
<td>82</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rheumatic fever</td>
<td>124</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tangier disease</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thalassemia</td>
<td>84</td>
<td>2</td>
<td>2.4</td>
</tr>
<tr>
<td>U. S. normal population**</td>
<td>1,524</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>2,907</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

* Fifty rheumatoid arthritis, 3 psoriatic, 4 Sjögren’s, 8 ankylosing spondylitis, 5 “connective tissue” disease.
† Includes 96 from the United States and 207 from Brazil.
‡ Includes 75 male patients from the New Jersey institution, and 5 females and 4 males from the Maryland institution.
¶ These sera were from patients suspected of having infectious or serum hepatitis.
§ One patient with Au(1) had both chronic lymphocytic leukemia and multiple myeloma and is included in both categories.
** This includes 607 Negro and 917 white persons.
addition, a single patient with Fanconi's anemia (hypoplasia of the marrow) and one patient (from Finland) with Hodgkin's disease have the antigen (Table 1).

The distribution of Australia antigen in leukemia and related disease groups is given in Table 2. The frequency is relatively high in acute granulocytic leukemia patients and to this date has not been found in any patient with chronic granulocytic leukemia. Australia antigen was found in a single case of the relatively rare chronic reticuloendotheliosis. None of the polycythemia vera patients had detectable Australia antigen.

We have recently concluded a study on Cebu Island, the Philippines, which included blood specimens from 584 patients with lepromatous leprosy, 377 patients with tuberculoid leprosy, and 764 controls without leprosy (19, 20). There was a significantly higher incidence of Au(I) in the lepromatous leprosy cases as compared with either the tuberculoid leprosy cases or the controls (9.4% lepromatous, 3.4% tuberculoid, 4.8% control). This difference is even more striking if only young men (under 20 years) are considered (26.7% lepromatous, 7.9% tuberculoid, 5.5% controls). As noted above, Au(I) is relatively common (about 5%) in nonhospitalized, apparently normal populations in several Asian and Oceanic countries including the Philippines (8).

### Table 2. Distribution of Australia Antigen in Leukemia and Related Diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Total No. of Cases</th>
<th>Positive for Australia Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute granulocytic leukemia</td>
<td>38</td>
<td>7</td>
</tr>
<tr>
<td>Chronic granulocytic leukemia</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>Acute lymphocytic leukemia</td>
<td>58</td>
<td>2</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Other*</td>
<td>10</td>
<td>2†</td>
</tr>
</tbody>
</table>

* Lymphosarcoma, 3, reticulum cell sarcoma, 3, chronic reticuloendotheliosis, 1, plasma cell leukemia, 1, acute stem cell leukemia, 2.
† Chronic reticuloendotheliosis, 1, acute stem cell leukemia, 1.

### Table 3. The Distribution of Australia Antigen by Age in Patients with Down's Syndrome and in Three Other Populations

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Positive</td>
<td>Cases</td>
<td>Positive</td>
</tr>
<tr>
<td>yr</td>
<td>no.</td>
<td>no.</td>
<td>%</td>
<td>no.</td>
</tr>
<tr>
<td>0–19</td>
<td>34</td>
<td>12</td>
<td>35.3</td>
<td>196</td>
</tr>
<tr>
<td>20–39</td>
<td>30</td>
<td>6</td>
<td>20.0</td>
<td>148</td>
</tr>
<tr>
<td>40–59</td>
<td>11</td>
<td>1</td>
<td>9.1</td>
<td>85</td>
</tr>
<tr>
<td>60+</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>67</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>19</td>
<td>—</td>
<td>496</td>
</tr>
</tbody>
</table>

* U.S. Trust Territory of the Pacific Islands.
† The Cebu population includes persons with and without leprosy.
TABLE 4. Blood Factors Studied in Australia Antigen-positive (Au(1)) and -negative (Au(0)) Sera*

<table>
<thead>
<tr>
<th>Study†</th>
<th>Positive, Au(1)</th>
<th>Negative, Au(0)</th>
<th>Total Au(1)+Au(0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Studied</td>
<td>Mean Value</td>
<td>No. Studied</td>
</tr>
<tr>
<td>Hemoglobin, g/100 ml</td>
<td>19</td>
<td>15.2</td>
<td>26</td>
</tr>
<tr>
<td>RBC count, million/mm³</td>
<td>19</td>
<td>4.48</td>
<td>26</td>
</tr>
<tr>
<td>Hematocrit reading, ‑</td>
<td>19</td>
<td>43.0</td>
<td>26</td>
</tr>
<tr>
<td>MCV, µl</td>
<td>19</td>
<td>96.2</td>
<td>26</td>
</tr>
<tr>
<td>MCH, µg</td>
<td>19</td>
<td>33.9</td>
<td>26</td>
</tr>
<tr>
<td>MCHC, ‑</td>
<td>19</td>
<td>35.3</td>
<td>26</td>
</tr>
<tr>
<td>WBC count, /mm³</td>
<td>19</td>
<td>7,153</td>
<td>26</td>
</tr>
<tr>
<td>Polymorphonuclear cells, ‑</td>
<td>19</td>
<td>56.3</td>
<td>26</td>
</tr>
<tr>
<td>Stab cells, ‑</td>
<td>19</td>
<td>4.1</td>
<td>26</td>
</tr>
<tr>
<td>Lymphocytes, ‑</td>
<td>19</td>
<td>3.4</td>
<td>26</td>
</tr>
<tr>
<td>Monocytes, ‑</td>
<td>19</td>
<td>4.1</td>
<td>26</td>
</tr>
<tr>
<td>Basophils, ‑</td>
<td>19</td>
<td>0.4</td>
<td>26</td>
</tr>
<tr>
<td>WBC G-6-PD, µmoles TPX reduced/10⁹ WBC/min</td>
<td>9</td>
<td>17.1</td>
<td>9</td>
</tr>
<tr>
<td>WBC G-1-PUT, µmoles UDPG/hr/10⁹ WBC</td>
<td>9</td>
<td>29.1</td>
<td>10</td>
</tr>
<tr>
<td>Platelets, /mm³</td>
<td>19</td>
<td>191,158</td>
<td>26</td>
</tr>
<tr>
<td>Uric acid, mg/100 ml</td>
<td>18</td>
<td>5.2</td>
<td>26</td>
</tr>
<tr>
<td>Vitamin B₁₂, µg/ml</td>
<td>17</td>
<td>698.6</td>
<td>23</td>
</tr>
</tbody>
</table>

* None of the differences were significant with the exception of vitamin B₁₂, where t = 2.926 and P < 0.01.
† MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, G-6-PD = glucose-6-phosphate dehydrogenase, TPX = triphosphopyridine nucleotide, G-1-PUT = galactose-1-phosphate uridyl transferase, UDPG = uridine diphosphate glucose.

are statistically significant for the Cebu population (0.02 > P > 0.01) and nearly so for the Marshall Islands (0.1 > P > 0.05) but not for the others.

CHROMOSOME STUDIES

Seventeen patients of the 75 Down's syndrome patients included in this study were selected for cytogenetic examination. Criteria used in the selection were low maternal age at birth or other indication of possible familial transmission of the syndrome, relatively high intelligence, and, in one case (385H), the relatively advanced age of the patient (60 years). One case was chosen at random. The selection was made without prior knowledge of the antigen phenotype. A minimum number of 10 metaphase chromosome counts and 1 karyotype analysis was made on each individual (leukocyte cultures); in those cases in which the modal chromosome number was not 47, additional counts and analyses were made. Fourteen of the 17 patients were found to be trisomic for chromosome 21, the classical finding in Down's syndrome (21). Of the remaining 3, 1 (389H) was a mosaic of normal and trisomy-21 cells, the second (384H) was the carrier of a 13-15/21 translocation and effectively trisomic for 21, and the third (385) had only chromosomes that were entirely normal in number and morphology in the single cell type studied. More extensive cytogenetic analysis might have revealed additional mosaicism in which cells of subordinate clones were present in low frequency, but because it is possible to study the chromosomes of relatively few cell types from a limited number of sites, it is impossible to rule out mosaicism.
completely in any human. None of these three had detectable Au(l).

Of the 14 typical Down's syndrome patients (that is, with 47 chromosomes), 3 had Australia antigen. Chromosome studies were done on 4 of the control patients. None of these had the typical Down's syndrome trisomy pattern.

The blood factors studies are shown in Table 4. With the exception of the vitamin B₁₂ values, there was no significant difference between the patients with and those without Australia antigen. The mean of the B₁₂ values for the positive group (Australia antigen present) was 698.6 and for the negative group (Australia antigen absent), 341.3. This gives a t value of 2.926 and 0.01 > P > 0.001 and indicates that it is highly unlikely that this difference between the means is due to chance. The levels of white blood cell galactose-1-phosphate uridyl transferase and glucose-6-phosphate dehydrogenase are higher on the average in the Au(l) than the Au(0) patients, but this difference is not significant. They are considerably higher than the values given for normal white blood cells in the table published by Mellman and colleagues (13). The hematocrit reading, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration for all the Down's syndrome patients combined are slightly higher than those for normals, confirming the findings of Naiman, Oski, and Mellman (22). The average of the uric acid levels for all patients combined is about the same as that found for male Down's syndrome patients by Mertz, Fuller, and Concon (23) (who used a different technique for measuring uric acid). They noted that young mongoloids had higher levels than did the control patients.

**DISCUSSION**

There is a significantly higher frequency of Au(l) in the Down's syndrome patients than in the controls. From this, we may conclude that the high frequency is associated with Down's syndrome and that it is not due to institutionalization alone. The frequency appears to decrease with age in the Down's syndrome patients as it does in other populations. There is a higher average vitamin B₁₂ level in the Au(l) individuals compared with the Au(0), but this finding should be confirmed before further comment is warranted. All the Down's syndrome patients with Au(l) and whose chromosome complement is known have the usual 21 trisomy.

The present state of knowledge concerning Australia antigen has been summarized in the introduction. The most striking feature of our data is the discovery of Australia antigen in high frequency in patients with Down's syndrome, leukemia (particularly acute granulocytic leukemia and chronic lymphocytic leukemia), and viral hepatitis. These diseases have been linked for other reasons: Patients with Down's syndrome have an increased risk of developing leukemia, particularly acute myelogenous leukemia (11); an association has been found between epidemics of hepatitis and the birth 9 months later of children with Down's syndrome (24); furthermore, it has been reported that sera of patients convalescing from hepatitis cause chromosome aberrations in tissue cultures (25).

Most of the disease associations could be explained by the association of Au(l) with a virus, as suggested in our previous publications (2, 6, 8). The discovery of the frequent occurrence of Au(l) in patients with virus hepatitis raises the possibility that the agent present in some cases of this disease may be Australia antigen or be responsible for its presence. The presence of Australia antigen in the thalassemia and hemophilia patients could be due to virus introduced by transfusions. This, however, could not be the only explanation, since many transfused patients neither have the antigen nor an antibody against it. To our knowledge,
none of the Down's patients with Australia antigen have received transfusions, and its presence in these patients may be the result of very early or maternal infection. Associations between Down's syndrome and thyroiditis (20) and rubella (27) have also been suggested, but these have not been studied in respect to Au(1).

As noted in the introduction, a striking family clustering of Au(1) has been found in Filipino and other populations (8). This could be explained on a simple infection hypothesis if the attack rate were higher in children than it is in adults. (See Table 3 of this paper and Table 5 of reference 8.) However, the segregation in the families is consistent with simple autosomal recessive inheritance. If Au(1) is inherited, as these results suggest, this would require an explanation consistent with the infection theory. It is possible that the trait is inherited in some groups such as the Filipinos but not in others. Or, the presence of the appropriate genes may confer an added susceptibility to infection with the virus. In areas where infection is very common, the trait could follow a pattern of mendelian segregation as it appeared to do in the Philippine studies.

Studies on the biochemical and immunologic nature of Au(1) are in progress, as are additional studies on the association with hepatitis. When the results of these are available, a more explicit interpretation may be possible.

Summary

Australia antigen (Au(1)) found in patients with leukemia (particularly those with acute granulocytic leukemia) has now been found in high frequency in patients with Down's syndrome and virus hepatitis. Down's syndrome patients with Australia antigen have, on the average, a higher level of vitamin B₁₂ than do the patients without this trait. Several hypotheses are discussed in an attempt to explain the associations.

Acknowledgments

This study was made possible as a result of the enthusiastic support and encouragement of Mr. F. W. Russell, Superintendent, and Dr. J. D. Nelson, Medical Director, of the State Colony at New Lisbon, New Lisbon, N. J. We are also indebted to the residents of the colony who participated in this study and to their parents, who have shown a continuing interest in the progress of our work.

Many of the valuable sera of patients used in this work were presented to us by physicians from the Philadelphia area and elsewhere to whom we are grateful. These include Drs. Erlandson, Erslev, Freireich, Frost, Joseph, Lawrence, Lee, McElfresh, Naiman, Osgood, Ultman, Whereat, Winchell, Woldow, and Wolman. We are particularly indebted to Dr. James E. Prier, Director, Division of Laboratories, Pennsylvania Department of Health, for supplying serum specimens from patients suspected of having viral hepatitis.

SUMMARIO IN INTERLINGUA

Antigeno Australia, trovate in patientes con leucemia (particularmente in casos de acute leucemia granulocytic) ha esiste incontrate con un alte frequenzia in patientes con syndrome de Down e hepatitis virusal. Patientes con syndrome de Down e antigeno Australia ha, al media, un plus alte nivello de vitamina B₁₂ que patientes sin iste tracto. Es commentate plure hypotheses presentate pro explicar le mentionate associationes.

References

Anicteric Hepatitis Associated With Australia Antigen

Occurrence in Patients With Down’s Syndrome

Alton I. Sutnick, MD; W. Thomas London, MD; Betty Jane S. Gerstley, MD; Malcolm M. Cronlund, MS; and Baruch S. Blumberg, MD, PhD

The frequency of Australia antigen is high in patients with Down’s syndrome in large institutions (27.7%) and is low in patients without this syndrome in the same institutions (3.2%). It is rare in patients with Down’s syndrome in small institutions (1.5%) and absent in newborn patients and those who are not institutionalized. Serum glutamic pyruvic transaminase levels were studied in age- and sex-matched groups of 16 patients with Down’s syndrome and Australia antigen [phenotype Au (1)], 16 with Down’s syndrome but without Australia antigen [Au(0)] and 16 Au(0) mental defectives without Down’s syndrome. The findings indicated active liver cell breakdown in Au(1) patients, and were confirmed in an extensive study including 581 individuals. The findings are best explained by the operation of an environmental factor, probably infectious, present in the large institutions, and a host susceptibility factor present in association with Down’s syndrome.

In recent reports we have described the characteristics of an unusual antigen present in the blood of certain people. The antigen was originally detected by micro-Ouchterlony technique with a precipitating antibody found in the blood of a transfused hemophiliac (Fig 1). It was termed “Australia antigen,” since it was first described as appearing in the serum of an Australian aborigine. It is extremely rare in normal Americans (two in 2,412) but is relatively common in patients with acute myelogenous leukemia (18.4%), chronic lymphocytic leukemia (13.3%), Down’s syndrome (9.4 to 29.8%), and nodular (lepromatous) leprosy (9.4%). We have been searching for features in common among this apparently heterogeneous group of diseases. The discovery of a strong statistical association between Australia antigen and viral hepatitis and the identification of Australia antigen as a particle suggest that hepatitis might be the common factor leading to the high frequency of Australia antigen in persons with these diseases.

In this communication we describe our studies of serum glutamic pyruvic transaminase (SGPT) levels in patients with Down’s syndrome, with and without Australia antigen, in institutions of various sizes and in patients living at home.

Methods

This study included 377 patients with Down’s syndrome; 34 were not institutionalized, and the rest were from nine separate institutions. Four were large state institutions for mental defectives, and five were small, private institutions. Institution 1 (State Colony at New Lisbon, NJ) has about 1,200 male residents, all of whom are 6 years of age or older. All of the 144 patients with Down’s syndrome in the institution were tested. The diagnosis of Down’s syndrome had been made clinically prior to admission. The patients with this syndrome are not housed separately, but live with other residents in several cottages. All patients eat in a common dining hall. Institution 2 (Pennhurst State School and Hospital, Spring City, Pa) has a total population of about 3,000 mentally retarded patients. About 250 have Down’s syndrome, of whom 113 have been tested. The clinical diagnosis is made at the admission examination. The patients with this syndrome are not housed separately, but live with other residents in different cottages. All patients eat in a common dining hall. The other two state institutions were of similar size. Each of the five private institutions had total patient populations of less than 50.

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From institutions 1 and 2, 188 mentally defective without Down's syndrome were tested. This included 76 patients from institution 1 who were classified as “epileptics” and 112 from institution 2 with various classifications of mental retardation. The testing also included 114 staff personnel from institution 2. Sera from 14 newborn patients with Down’s syndrome, confirmed by cytogenetic studies, were tested. In addition, 20 patients with Down’s syndrome who were not institutionalized were tested. Fourteen of these attended the day school of the Philadelphia Association for Retarded Children (Table 1).

Table 1.—Distribution of Australia Antigen in Mentally Retarded Patients and Institution Staff Personnel

<table>
<thead>
<tr>
<th>Group Tested</th>
<th>No. Tested</th>
<th>No. Positive</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down's syndrome (large institutions)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Institution 1 (all males over 6 years old)</td>
<td>144</td>
<td>41</td>
<td>28.5</td>
</tr>
<tr>
<td>Male</td>
<td>68</td>
<td>26</td>
<td>38.2</td>
</tr>
<tr>
<td>Female</td>
<td>48</td>
<td>8</td>
<td>17.8</td>
</tr>
<tr>
<td>Two other large institutions</td>
<td>53</td>
<td>11</td>
<td>20.8</td>
</tr>
<tr>
<td>Total</td>
<td>310</td>
<td>86</td>
<td>27.7</td>
</tr>
<tr>
<td>Down's syndrome (other)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Five small institutions</td>
<td>33</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Patients who were not institutionalized</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Newborn</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Mentally retarded (unclassified, from large institutions)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Institution 1</td>
<td>76</td>
<td>4</td>
<td>5.3</td>
</tr>
<tr>
<td>Male</td>
<td>69</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>Female</td>
<td>43</td>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td>Total</td>
<td>188</td>
<td>6</td>
<td>3.2</td>
</tr>
<tr>
<td>Staff personnel (large institution)</td>
<td>114</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Sera were tested for Australia antigen with both human and rabbit antisera by the micro-Ouchterlony technique of Blumberg and Riddell. Quantitative studies were done with the methods of double diffusion in agar gel tubes described by Preer and Preer. The SGPT level was determined by the method of Wroblewski and La Due at 89.6 F (32 C) with a recording spectrophotometer. Sera were stored at -13 F (-25 C) until the SGPT measurements were completed. All SGPT determinations were completed within seven days of drawing the blood. The enzyme is known to be stable at this temperature for this period of time.

Liver biopsy was performed for diagnostic purposes on four patients with Down’s syndrome, Australia antigen, and an elevated SGPT level. These were open biopsies, done with the patients under general anesthesia, because adequate cooperation could not be obtained for a needle biopsy. Because of the element of risk involved, only those patients in whom the procedure was of clinical diagnostic importance and for whom proper consent from informed parents or guardians could be obtained, were subjected to this procedure. The specimens were fixed in formaldehyde solution, stained with hematoxylin and eosin, and examined by light microscopy.
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2. Serum glutamic pyruvic transaminase (SGPT) levels in age- and sex-matched groups of patients with Down's syndrome [Au(l) and Au(0)], and other mental defects [Au(0)]. Medians and range indicated.

Statistical comparisons were done with Student's $t$ test and an MN Chi computer program, providing an age- and sex-corrected comparison of two groups regarding frequency of the Au(1) phenotype.

**Results**

Australia antigen is common in patients with Down's syndrome in large institutions, and is extremely rare in patients without Down's syndrome in the same institutions (Table 1). The difference is highly significant when an age- and sex-corrected comparison is made with the MN Chi program ($P < 0.001$). This confirms our previously reported findings. The frequency is much lower in smaller institutions. It is entirely absent in newborn patients with Down's syndrome, in those who live at home, and in the staff personnel of institution 2 (Table 1). The frequency of Australia antigen is age-dependent in the Down's syndrome group (highest frequency in the youngest patients), as it is in other populations which have been tested. It is also more common in males than in females, but this difference is not significant when an age- and sex-comparison is made with the MN Chi program ($P = 0.1057$). A similar sex-related difference has been reported in other populations.

There is a clear association between the presence of Australia antigen and an elevated SGPT level (Table 2). This is seen in the age- and sex-matched comparison in Fig 2 [Down's Au(1), 38.7 units/ml; Down's Au(0), 14 units/ml; and non-Down's Au(0), 16 units/ml], and in the subsequent larger study [Down's Au(1), 39.4 units/ml; Down's Au(0), 15.4 units/ml; and non-Down's Au(0), 14.6 units/ml]. The findings are the same in both institutions (Fig 3). The association is so close that, in general, the presence of Australia antigen can be used as an indication of an elevated SGPT level. The median SGPT values in the patients with Down's syndrome who were not institutionalized (11.5 units/ml) and the staff (13 units/ml) are also significantly lower than the levels in the Au(1) patients with Down's syndrome.

Inflammatory changes of various degrees were found in the liver of each of the four patients who had Down's syndrome, Australia antigen, an elevated SGPT level, and who had a biopsy performed. Patient 1 had periportal inflammation with lymphocytes and plasma cells, minimal hydropic change and binucleation of liver cells, and an increase in the number of Kupffer's cells with the presence of ceroid pigment. Patient 2 showed marked hydropic degeneration with balloon-like distension of the cells, liver cell breakdown with loss of margin, cellular debris, and periportal infiltration of lymphocytes and plasma cells (Fig 4, top). In this patient, there was also nodule formation indicative of post-necrotic cirrhosis (Fig 4, bottom). Patient 3 had increased collagenous connective and fibrous tissue in the hepatic triads, and infiltration by lymphocytes and plasma cells. There was bile duct proliferation and focal liver cell necrosis with cytoplasmic degeneration, acidophilic bodies, and cellular reactive infiltrate. The Kupffer's cells contained ceroid pigment and blue-staining granular matter which has remained unidentified. Patient 4 showed periportal lymphocyte, plasma cell, and histiocytic infiltration, advanced albuminous degeneration.
large nuclei, irregular liver cell size, cell destruction, and cellular debris. Histologically, this appeared to be a more active case of hepatitis.

**Comment**

Australia antigen is associated with an elevated SGPT value in patients with Down's syndrome. The four Au(1) patients with elevated SGPT levels who underwent biopsy had histological evidence of hepatitis. There was a small number of liver biopsies performed, and no control biopsies were done in patients without Australia antigen. Consequently, this cannot be considered proof that Australia antigen is associated with liver damage, but is consistent with this hypothesis. The antigen, with a concomitant raised SGPT value, is rare or absent in other mental retardates and in staff personnel from the same institutions. High SGPT levels are due to release of the enzyme as a result of liver cell damage or death. Australia antigen thus appears closely related to some agent responsible for liver cell injury.

The increased prevalence of Australia antigen in patients with Down's syndrome in large institutions is striking. The confirmation of the clinical diagnosis in all of the 82 patients whose chromosome preparations were studied makes it seem unlikely that major discrepancies occur in the remaining patients. Even if several erroneous diagnoses were included, the statistical correlation is so great that the conclusions would remain unchanged. Although the frequency of the antigen in patients with Down's syndrome is high in large institutions, it has not been found in patients who are not institutionalized and occurs in very low frequency in small institutions. (In addition to the 20 sera we examined of patients with Down's syndrome who were not placed in institutions, L. J. Old, MD, of New York told us [in June 1967] that Australia antigen was not found in sera from 25 such patients. These sera were collected by J. Storm, MD, of Manhasset, NY.) This suggests that a factor present in large institutions may be responsible for the presence of Australia antigen. It is reasonable to suppose that this factor is an infectious agent, such as a virus, which involves the liver and is disseminated by frequent and intimate contacts between individuals in large and crowded populations. Many chemicals and drugs may cause liver damage, but these seem less likely to be implicated in this situation. There is additional evidence consistent with the hypothesis of infection. Australia antigen clusters in families. It occurs in high frequency in tropical areas where the people have poor sanitary practices, and its age-related distribution (higher frequency in younger than in older people) is similar to the attack rate of viral hepatitis.

The following are two remarkable features of these findings: (1) limited distribution of the antigen in institutions, occurring nearly exclusively in patients with Down's syndrome, and (2) absence of the antigen in patients with Down's syndrome who live at home. This strongly suggests that the following two factors are in operation: (1) an environmental factor, for example, an infectious agent, present in the large institutions, and (2) a host factor which makes the patients with Down's syndrome more susceptible than other inhabitants of the same environment. Patients with Down's syndrome are either more susceptible to infection with the postulated agent or retain the agent chronically. In either event, this implies impairment of the immune mechanism in patients with Down's syn-
drome. Inherited susceptibility to this postulated agent may explain previously reported family data which are consistent with simple autosomal recessive inheritance of Australia antigen.

Australia antigen is also commonly associated with other chromosome abnormalities. Four of 16 patients with a variety of autosomal and sex chromosome abnormalities have the antigen (unpublished observations). Leukemia is also relatively common in patients with Down’s syndrome and in patients with certain other chromosome abnormalities. We have previously reported the high frequency of Australia antigen associated with some types of leukemia. The definition of the mechanism linking these findings is the subject of our current research.

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References


Australia Antigen and Acute Viral Hepatitis


Philadelphia, Pennsylvania

Summary

Australia antigen (Au(I)) was found in the sera of 20% of 125 patients with acute viral hepatitis. It was not present in the sera of 138 patients with other diseases affecting the liver (Laennec's cirrhosis, infectious mononucleosis, hepatoma, and so on). These data indicate that the antigen is selectively associated with viral hepatitis and is not a nonspecific manifestation of liver damage.

Previously we have reported the association of Au(I) with leukemia, lepromatous leprosy, and Down's syndrome, and in 1 to 20% of some tropical populations. In these groups the antigen occurs more frequently in young males; once present, it tends to persist for long periods (years). In the acute hepatitis patients there was no significant difference in distribution between the sexes; the antigen was not found in any patient less than 19 years old, and with two exceptions it was present in the sera for short intervals (days and weeks). Host factors related to immunologic competence may be responsible for these differences.

We have previously described the characteristics of an unusual antigen, Australia antigen (Au(I)) (1–3). When present in serum the antigen forms a precipitin with specific human and rabbit antisera (4). With the use of the micro-Ouchterlony technique the sera from large numbers of persons with and without disease have been tested. The antigen occurs frequently in tropical populations (1 to 20%) but rarely in normal North Americans and Europeans (2). In the United States we have found increased frequencies of the antigen in association with certain diseases, that is, acute and chronic lymphocytic leukemias, acute granulocytic leukemia, Down's syndrome, and viral hepatitis (3).

In the same U. S. study those patients with Down's syndrome and Australia antigen had significantly higher levels (mean and median) of serum glutamic-pyruvic transaminase (SGPT) than did age- and sex-matched patients with Down's syndrome without the antigen. Liver biopsies from eight such patients with Au(I) showed chronic hepatitis (5).

The antigen has been isolated from the sera of five patients by ultracentrifugation on a sucrose gradient. Electron microscopy showed that these isolates contained particles 20 mμ in diameter, and these agglutinated upon the addition of specific antisera against Au(I) (6).

We have considered two alternative hypotheses to explain the disease associations and physical characteristics of Australia antigen: [1] Australia antigen is released into the circulation as a result of liver injury and its presence in serum is a nonspecific reflection of hepatic damage; and [2] Australia antigen is intimately associated with a virus causing hepatitis, and its presence in serum indicates the presence of that virus. Results compatible with the second hypothesis are reported here.

Materials and Methods

Serum was collected from 125 patients with viral hepatitis and 2 or more samples were ob-

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Requests for reprints should be addressed to W. Thomas London, M.D., The Institute for Cancer Research, 7701 Burholme Ave., Fox Chase, Philadelphia, Pa. 19111
tained from 56 of these patients. Seventy-five sera were provided by Drs. Prier and Strachen of the Pennsylvania State Department of Health Laboratories. The remainder were obtained from patients at affiliated hospitals of the University of Pennsylvania; the Jeanes Hospital, Fox Chase, Philadelphia; and Bellevue Hospital, N. Y. In the hospital group the diagnosis of hepatitis was established by serum glutamic-pyruvic transaminase (SGPT) elevation, or liver biopsy, or both. With respect to the sera obtained by the State Department of Health, we attempted to substantiate the diagnosis by contacting the patients’ physicians 6 months to a year after the acute illness. The patients included here are those in whom the diagnosis was confirmed. Of the 125 patients, 41 had received a prior transfusion or were considered by their physicians to have had serum hepatitis; we have classified these as posttransfusion hepatitis and all the remaining cases as infectious hepatitis.

Sera were obtained from 57 patients with Laennec’s cirrhosis at the affiliated hospitals of the University of Pennsylvania and at the Veterans Administration Hospital, Brooklyn, N. Y. Sera of 55 patients with heterophil positive infectious mononucleosis (IM) were kindly provided by Dr. Esko Kaarsalo of Turku, Finland. An additional 12 sera from patients with IM were collected at the Student Health Clinic of the University of Pennsylvania. Sera from 14 patients with other diseases involving the liver were tested. These included three patients with granulomatous hepatitis, two with jaundice secondary to choledocholithiasis, one with carcinoma of the common bile duct, five with hepatoma, two with acute cholecystitis, and one with Wilson’s disease. All of these were patients at hospitals in Philadelphia; the diagnoses were made on aspiration liver biopsy or at laparotomy with biopsy.

In order to obtain an estimate of the frequency of the antigen in the normal population of the United States, 2,120 sera from presumed normal persons were tested. Of these, 563 (323 Negroes and 240 whites, ages 15 to 74 years) were from a sample of the population of Evans County, Ga. (7); 215 were white employees of the National Institutes of Health, Bethesda, Md.; 180 were negro employees of the same institution; 80 were white residents of Tangier Island, Md., and 187 were negro residents of Sapelo Island, Ga. Others included were 518 blood donors at Jeanes Hospital, 114 members of the staff of Pennhurst State School and Hospital, Spring City, Pa., 42 sets of twins from Evans County, Ga., 43 members of the staff of the Institute for Cancer Research, Philadelphia, Pa., and their families, and 105 specimens from various other normal persons.

A broad sample of the patient population in Philadelphia was obtained by testing 974 consecutive sera (486 individuals) collected at the clinical laboratory of the Jeanes Hospital and 1,064 consecutive sera (556 individuals) collected at the Pepper Laboratory of the Hospital of the University of Pennsylvania.

The sources of patients with Down’s syndrome (Tables 2, 3, and 4) have been reported in detail previously (5).

All sera were tested for Australia antigen with specific rabbit antiserum (4) by the micro-Ouchterlony technique of Blumberg and Riddell (8). Semiquantitative estimates of the titer of Au(l) were performed by double diffusion in capillary tubes using the method of Preer and Preer (9). Contingency tables and chi-square determinations were done as described by Snedecor (10).

**Table 1. Frequency of Australia Antigen (Au(l)) in the United States in Patients with Viral Hepatitis, and Other Liver Diseases and in Controls**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Au(l)</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>U. S. normal population</td>
<td>2,120</td>
<td>2</td>
<td>0.09</td>
</tr>
<tr>
<td>U. S. hospital inpatients</td>
<td>1,024</td>
<td>1</td>
<td>0.19</td>
</tr>
<tr>
<td>Infectious mononucleosis</td>
<td>67</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Laennec’s cirrhosis</td>
<td>57</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other liver diseases</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Viral hepatitis</td>
<td>125</td>
<td>25</td>
<td>20.0</td>
</tr>
<tr>
<td>Infectious</td>
<td>84</td>
<td>11</td>
<td>13.1</td>
</tr>
<tr>
<td>Posttransfusion</td>
<td>41</td>
<td>14</td>
<td>34.1</td>
</tr>
</tbody>
</table>

The results of 125 patients with hepatitis had Au(l) on one or more occasions (Table 1), whereas Australia antigen occurs in less than 0.1% of the normal population. (Of the two presumed normal persons with Au(l), one had had a previous transfusion and the other had been exposed to hepatitis.) The only hospitalized patient with Au(l) had acute posttransfusion hepatitis at the time her blood was drawn. The antigen was not found in 138 patients with liver diseases other than viral hepatitis. Of particular significance is its absence in the
Table 2. Frequency of Australia Antigen (Au(l)) by Sex in Patients with Viral Hepatitis and Institutionalized Patients with Down's Syndrome

<table>
<thead>
<tr>
<th></th>
<th>Hepatitis</th>
<th>Down's Syndrome*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Au(l)</td>
<td>Percent Au(l)</td>
</tr>
<tr>
<td></td>
<td>no.</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>63</td>
<td>14</td>
</tr>
<tr>
<td>Female</td>
<td>62</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>125</td>
<td>25</td>
</tr>
</tbody>
</table>

* From two Institutions housing both males and females. (X² = 10.97, P = <0.025).

Infectious mononucleosis group, since these patients have a type of hepatitis thought to be caused by a virus (11).

The hepatitis patients are divided into two categories: [1] posttransfusion hepatitis—those who had a prior transfusion or were considered by their physicians to have serum hepatitis; and [2] infectious hepatitis—the remaining hepatitis cases. The difference in the frequencies of Au(l) in posttransfusion hepatitis (34.1%) and infectious hepatitis (13.1%) is statistically significant (X² = 6.46, P < 0.025).

Other populations with an increased prevalence of Au(l) (Down's syndrome, lepromatous leprosy, and "normal" persons from the Philippines and Micronesia) have higher frequencies of the antigen in males than in females and higher frequencies in younger than in older persons (2, 5). Patients with Down's syndrome who are institutionalized are the most thoroughly studied of such populations in the United States (5) and have been used here for comparison with the hepatitis patients, but patients with lepromatous leprosy and "normal" populations from the Philippines and Micronesia show approximately the same distributions of Au(l) (2, 9). In the hepatitis patients the frequency of Au(l) is essentially the same in both sexes: 22% in males and 18% in females. This distribution of Au(l) by sex is significantly different from that found in institutionalized patients with Down's syndrome where the prevalence in males is almost twice that in females (X² = 10.97, P = <0.025, Table 2).

Table 3 shows the distribution of Au(l) by age in the hepatitis patients and institutionalized patients with Down's syndrome. Australia antigen has not been found in any hepatitis patient younger than 19 years old, whereas among the patients with Down's syndrome the highest frequency of the antigen (43.3%) is in the 0 to 19 year age group. These distributions differ significantly (X² = 20.77, P < 0.005).

Another characteristic of Australia antigen in other diseases and population groups is its persistence (2, 5). In hepatitis, however, the antigen is transient. In Table 4 the duration of Au(l) in patients with hepatitis and patients with Down's syndrome is compared. Of 15 patients with hepatitis and Au(l) on first testing, the antigen disappeared from 13 after 2 to 3 weeks. In one patient Au(l) was present for only a single day, but another hepatitis patient had Au(l) for 7 months (his SGPT's remained over 100 during that time). In
contrast, of 12 patients with Down's syndrome and Au(l) on first testing, 10 still had the antigen when tested a year later. These differences in persistence are significant ($\chi^2 = 31.59$, $P < 0.001$). Figure 1 gives the variation of Au(l) titer, estimated by the Prer technique, and the SGPT levels in a patient with acute posttransfusion hepatitis. In this patient the Australia antigen titer parallels the fall in SGPT. Such a direct parallel between SGPT and Au(l) titer has not been found in patients with Down's syndrome.

**Discussion**

The data indicate that Australia antigen is associated with viral hepatitis, confirming our earlier studies (3, 12). Furthermore, Au(l) is not associated with other diseases affecting the liver; that is, the presence of the antigen in serum is not a nonspecific manifestation of liver damage.

Okochi and Murakami (13) have confirmed the association of Australia antigen with hepatitis in Japan. They found 44 of 358 patients with hepatitis to have Australia antigen. They also screened all of the blood and blood donors passing through the blood transfusion service of the Tokyo University Hospital and found 141 of 11,820 blood donors to have Australia antigen. SGPT levels were slightly but significantly higher in sera containing Au(l). They further demonstrated that persons receiving blood containing Australia antigen could either develop antibody to Au(l) or the antigen itself. Those in whom the antigen appeared had posttransfusion hepatitis.

We have pointed to a number of characteristics of Australia antigen in patients with hepatitis different from those in other groups where Au(l) is present in high frequency, such as Down's syndrome, lepromatous leprosy, and "normal" persons living in tropical areas. Persistence, higher frequency in males than females, and higher frequencies in the first two decades of life have been consistent findings in these groups. In contrast, transience, lack of sex predilection, and absence of Au(l) in patients under the age of 19 are features of the hepatitis group. The biologic meaning of these differences is not clear, but they may reflect the immunologic status of the host. That is, the patients who develop viral hepatitis do not have an underlying abnormality and are able, in a short time, to clear their blood of antigen and to effect complete recovery. The patients who retain the antigen chronically have an immunologic defect and therefore do not rid themselves of antigen nor do they completely eradicate the inflammatory process (14, 15).

The patients with Down's syndrome illustrate this hypothesis.

In large state institutions for the mentally retarded, viral hepatitis is a very common disease; probably every patient is exposed to the virus (16). Yet Australia antigen occurs much more frequently in the patients with Down's syndrome than in the other patients in the same institutions. Furthermore, patients with Down's syndrome and Au(l) have elevated SGPT's and have evidence of hepatitis on liver biopsy (5).

In this study we found a significantly higher frequency of Au(l) in posttransfusion hepatitis than in infectious hepatitis. Okochi and Murakami (13) did not find this difference. Further studies are necessary before we can determine whether the preva-
lence of Au(1) is greater in posttransfusion hepatitis.

Isolated Australia antigen is a particle 20 m\(\mu\) in diameter with an appearance compatible with that of a virus (6). Currently, we are inoculating animals and tissue cultures in an effort to determine whether it has other virus characteristics.

**Addendum**

Since the submission of this manuscript, an additional confirmation of the association of hepatitis with Australia antigen has appeared. The SH antigen described by Prince (17, 18) is Australia antigen.

**Acknowledgments**

We wish to thank Dr. J. Prior and Dr. S. Struchen (Pennsylvania State Department of Health Laboratories) for sera from hepatitis patients, Dr. R. Donato (Jeanses Hospital), Dr. D. Arvan (Hospital of the University of Pennsylvania), Dr. E. Kaarsalo, and Dr. C. Hames for sera and diagnostic information.

**References**

HEPATITIS AND AUSTRALIA ANTIGEN:
AUTOSOMAL RECESSIVE INHERITANCE OF
SUSCEPTIBILITY TO INFECTION IN HUMANS*

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Abstract.—Examples of inherited susceptibility to infection controlled by
genes segregating at one or a small number of loci have been identified in lower
animals. In this study we report data on what appears to be a similar situation
in humans. “Australia antigen” is an antigen found in the sera of patients
with acute and chronic hepatitis, and it may actually be a form of virus. It
is very common in many tropical areas, and people in these areas having the
antigen appear to be hepatitis carriers. The antigen is detected by immuno¬
diffusion in agar gel (Ouchterlony method). Individuals with the antigen
are designated Au(1) and those without it Au(0). Family studies involving
1797 different individuals residing on the island of Bougainville are consistent
with the hypothesis that susceptibility to chronic infection with the “antigen”
is controlled by an autosomal recessive gene (Au'). This confirms the con¬
clusions previously arrived at from similar (but less extensive) studies on the
island of Cebu. Individuals with this inherited susceptibility do not ordinarily
have overt manifestations of hepatitis.

The sign test was used to determine family clustering. Segregation analysis
was performed by the method of C. A. B. Smith. In the 41 Au(0) × Au(0)
matings, 53.8 recessives were expected in the offspring and 56 were seen (0.7
> p > 0.5). In the Au(1) × Au(0) matings, 40.1 recessives were expected
and 42 were seen (0.7 > p > 0.5). The Au(0) × Au(0) matings were also
analyzed by the method of Li and Mantel, in which the recessive ratio of 0.25
is expected by the genetic hypothesis. The values observed were 0.2527 for
the Bougainville study and 0.2461 for the Cebu study.

Inherited resistance and susceptibility to infection has been described in
several animal species. There is a single gene-controlled factor which prevents
infection of mice by a group of related viruses including yellow fever, West
Nile fever, Japanese B encephalitis, and others but does not prevent infection
with other viruses. Gowen has described inherited resistance to bacterial
infection in mice and has emphasized the specific nature of the resistance which
develops. There is evidence for inherited susceptibility to several forms of
virus-induced neoplasms in animals. These include polyoma virus in mice,
in which two or three independent genes may be involved in the determination
of resistance; mammary tumor in mice; leukemia in mice, where the inherited
factor appears to be related to the histocompatibility locus; Rous sarcoma;
fowl leucoses; and others. In humans, several inherited diseases carry with
them an increased susceptibility to infection; for example, patients with the
inherited disease sickle-cell anemia are unusually susceptible to *Salmonella* infections, with osteomyelitis as a frequent complication, while the heterozygotes appear to have an increased resistance to falciparum malaria. Patients with inherited sex-linked agammaglobulinemia are highly susceptible to several bacterial infections. There is also evidence for differences in infectious disease susceptibility between different population groups (i.e., increased tuberculosis infection in American Indians and increased infection with eucocidiodomyxos in Filipinos and American Negroes) and some of this difference in susceptibility may be inherited. These have been reviewed recently.9-11

In this paper we will describe family studies of a serum factor closely associated with acute and chronic viral hepatitis. The segregation of the trait in the families is consistent with simple recessive autosomal inheritance, and the data can be interpreted as an example of increased susceptibility to a chronic virus infection in humans, apparently controlled by genes segregating at a single autosomal locus.

In 1966 we reported on the family clustering and segregation of a serum antigen present in high frequency in certain tropical populations.12 Because it had been first identified in an Australian aborigine, the antigen was termed "Australia antigen." In the family study we utilized data collected on the Visayan island of Cebu in the Philippines. In the 53 families studied, the antigen was more commonly found among relatives of individuals who had the antigen (17%) than among the relatives of those who did not have it (2%). The hypothesis of simple autosomal recessive inheritance was tested in the families in which the trait was present. (Inspection of pedigrees ruled out simple autosomal dominant and sex-linked inheritance.) Using the segregation analysis method of C. A. B. Smith13 (Table 3), we found a very close fit to the numbers predicted by the hypothesis; the data provided strong support for the hypothesis of simple autosomal recessive inheritance.

This initial evidence for the genetic determination of the presence of the antigen was interesting since we know that Australia antigen also has features of an infectious agent associated with hepatitis.14-17 This has been reviewed16, 17 and will be summarized here. Since 1961 we have systematically examined the sera of transfused patients for the presence of precipitating isoantibodies against constituents of human sera.18 With these antisera, the Ag system (inherited antigenic specificities on the low-density lipoproteins)19 was discovered. We subsequently found that patients who received large numbers of transfusions (particularly those with hemophilia) quite often develop specific antibodies against Australia antigen.20-22 Antisera may also be produced by immunizing rabbits with the serum of a patient with Australian antigen and absorbing with a serum which does not contain the antigen.23

The antigen occurs in high frequency in acute hepatitis,14-18 but in most cases it is transient (days or weeks). The association of hepatitis with Australia antigen has been confirmed by Okochi and Murakami,22 Prince24, 25 (the SH antigen of Prince is indistinguishable from Australia antigen), and Vierucci,26 with reference sera from our laboratory. It also occurs chronically (for months or years) in Down's syndrome patients,14, 27 in three forms of leukemia,14, 31 and
in apparently normal people in vast areas of Asia and Oceania as well as elsewhere in the tropics.20 In these groups of patients it appears to be associated with chronic anicteric hepatitis.22, 28, 29 The liver abnormality may be very slight, particularly in the case of the apparently normal tropical populations referred to.

As noted, Australia antigen is more common in the general populations in some tropical countries (6–25%) and in Japan (1%) than it is in the United States (0.1%). The studies by Okochi and Murakami22 in Japan show that Australia antigen may be transmitted by transfusion. Patients transfused with the blood of donors containing Australia antigen may develop the antigen in their own blood, along with hepatitis, or they may develop antibodies to Australia antigen with no evidence of disease. Hence, the detection of the antigen in donor bloods is useful in screening for hepatitis carriers. It has also proved very useful in detecting occult cases of hepatitis in patients with renal disease receiving chronic hemodialysis.30 The "Au test" is now being used for the diagnosis of viral hepatitis and for the detection of hepatitis carriers.

Studies on the Down's syndrome patients demonstrate that both environmental (infectious) and host (congenital) factors are operating to determine the presence of the antigen.17, 29 In large institutions, Australia antigen is very common in Down's syndrome patients but extremely rare in other mentally retarded children and in normal individuals. That is, there is a host factor in the Down's patients which makes them more susceptible to chronic infection with Australia antigen. Australia antigen was not found in Down's patients who live at home and attend day schools. Down's patients who are in-patients at small private institutions where sanitation and care are generally better have extremely low frequencies (3%) of the antigen. This is compatible with the explanation that some environmental factor, presumably an infection, is also operating and that the crowded conditions of large institutions lead to much higher frequencies of the antigen.

Australia antigen can be isolated by centrifugation in high-density sugar gradients. Isolated fractions examined under the electron microscope contain large numbers of particles of approximately 200 Å in diameter;31 this is about the size postulated for hepatitis virus. Although the appearance of the particles is consistent with that of a virus, this, of course, does not represent firm evidence that these particles are in fact virus.

All these data taken together (and briefly reviewed here) have led to the hypothesis that Australia antigen is or is associated with an infectious agent that occurs in viral hepatitis. The theoretical consequences of this will be discussed elsewhere.17

In view of these findings, it became extremely important to retest the genetic hypothesis by further family studies. Collections of sera on the island of Bougainville, Trust Territory of New Guinea, have made this possible. This paper contains a report of these studies.

During 1966–1967 one of us (J. F.) collected blood from individuals in 18 villages on Bougainville. In each village he attempted to collect blood from as large a number of individuals as possible. About 85% of the people over two
years old were included in the collection. The majority of these sera were made available for Australia antigen testing. These populations are described in detail elsewhere. A total of 1797 sera were tested, including 617 family groupings. ABO and Rh blood groups and serum haptoglobin types were available in the individuals whose serum had been tested. If the paternity of any of the offspring in a family was questioned by the use of these markers, then the entire family was removed from the calculations and data from these individuals do not appear in the tables given herein. The presence or absence of the antigen in the plasma was determined by immunodiffusion in agar gel by a micro-Ouchterlony method. In this, the antiserum against Australia antigen is placed in the center well of the Ouchterlony pattern and the sera to be tested in the peripheral wells. The presence of the antigen is indicated by the appearance of a precipitin line which stains red with azo carmine. Human antiserum from a hemophilia patient and an antiserum made by the immunization of a rabbit with a serum containing Au(1) were used. The sera to be tested were scored as Au(1) if a precipitin band was seen under these experimental conditions and as Au(0) if a precipitin band was not seen. After the sera were scored for the presence or absence of antigen, results were recorded on the pedigree charts. In this manner the individuals testing the sera were not aware of the family relationship and the scoring was objective.

Results and Discussion.—The frequency of Au(1) in the 18 villages varied from 2.9 to 23.9 per cent. In order to determine if there is family clustering for the trait, IBM punch cards were prepared for each of the 1797 sera, given sequential numbers, and sorted in the IBM 1620 with a random numbers program. The cards were then examined in turn until the card of an individual who was scored as Au(1) was found. The next card of an individual of the same sex and within five years of the same age, but who was scored as Au(0), was selected as a control. The cards of the immediate family (mother, father, daughter, son, brother, sister, grandmother, grandfather, grandson, granddaughter) of the individual scored Au(1) were then examined and the frequency of Au(1) was determined. The same was done for the family of the control (Au(0)) individual. Twenty-four pairs were studied in this manner.

The significance of the results was tested by the Sign test. If the frequency of Australia antigen was higher in the family of the Au(1) individual than in that of the control (Au(0)), it was scored as positive; if the reverse was true, as negative. If there was no clustering in the families of individuals with Australia antigen, then an equal number of positives and negatives would be expected; but this was not seen (Table 1). Twenty-one of the twenty-four pairs studied were positive, two were negative, and one was the same; and this is highly unlikely to be due to chance \(p < 0.001\). This difference was not due to the differences in Au(1) frequencies in the villages from which the case and controls were selected.

Inspection of the pedigrees indicated that if the trait were inherited, it could not be inherited as a simple autosomal dominant trait or as a sex-linked trait. A simple autosomal, recessive hypothesis, the same as that tested in the Cebu material, was also tested with the present material. This hypothesis states
Table 1. Clustering of Au(1) in families as shown by the Sign test.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>14.7</td>
<td>14.7</td>
<td>8</td>
<td>37.5</td>
<td>18</td>
<td>16.6</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>66.6</td>
<td>11</td>
<td>0</td>
<td>+</td>
<td>9</td>
<td>9.1</td>
<td>24</td>
<td>8.3</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>15.4</td>
<td>14</td>
<td>0</td>
<td>+</td>
<td>12</td>
<td>25.0</td>
<td>14</td>
<td>14.7</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>22.2</td>
<td>8</td>
<td>0</td>
<td>+</td>
<td>10</td>
<td>10.0</td>
<td>19</td>
<td>31.6</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>50.0</td>
<td>10</td>
<td>10.0</td>
<td>+</td>
<td>17</td>
<td>29.4</td>
<td>29</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>41.1</td>
<td>22</td>
<td>13.5</td>
<td>+</td>
<td>21</td>
<td>9.5</td>
<td>3</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>20.0</td>
<td>11</td>
<td>0</td>
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<td>21</td>
<td>9.5</td>
<td>13</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>40.0</td>
<td>8</td>
<td>37.5</td>
<td>+</td>
<td>33</td>
<td>21.2</td>
<td>13</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>10.5</td>
<td>8</td>
<td>0</td>
<td>+</td>
<td>27</td>
<td>25.8</td>
<td>16</td>
<td>12.5</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>50.0</td>
<td>8</td>
<td>0</td>
<td>+</td>
<td>9</td>
<td>44.4</td>
<td>15</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>27.2</td>
<td>34</td>
<td>20.6</td>
<td>+</td>
<td>9</td>
<td>11.1</td>
<td>13</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>6.7</td>
<td>10</td>
<td>30.0</td>
<td>-</td>
<td>17</td>
<td>11.7</td>
<td>13</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

The number of family members and the per cent with Au(1) in the family of Au(1) individuals are given in columns 1, 2, 6, and 7. The same data for individuals who are Au(0) are given in columns 3, 4, 8, and 9. When the percentage of Au(1) was greater in the families of the Au(1) individuals than it was in the families of the Au(0) individuals, the doublet was scored as positive; if the opposite was the case, as negative. There were 21 positives and 2 negatives; it is highly unlikely that this is due to chance ($p < 0.001$).

that in individuals homozygous for a gene designated Au<sup>1</sup> (i.e., genotype Au<sup>1</sup>/Au<sup>1</sup>) Australia antigen would be detectable by the Ouchterlony method (phenotype Au(1)). In individuals homozygous for the alternate gene (Au/Au) and heterozygotes (Au<sup>1</sup>/Au), Australia antigen (phenotype Au(0)) would not be detectable.

Two methods of segregation analysis were used to test the genetic hypothesis. In Smith's method, the Au(0) X Au(0) and Au(1) X Au(0) matings are considered separately and corrections are made for family size.

For the Au(1) X Au(0) matings the calculations of Smith's Table 1 are followed, and the results are shown here in the upper part of Table 2. If the genetic hypothesis is correct, then 40.1 recessives are expected, and 42 are seen. The variance (as defined by Smith) is 11.0, the $\chi^2 = 0.341$ and $0.7 > p > 0.5$. This is a good fit to the expected. Included in this calculation are ten families in each of which one parent was positive and the other untested. It was assumed in each case that the untested parent was Au(0). The probability of a positive by positive mating is about 1/100 in this population.

The calculations for the Au(0) X Au(0) matings, which follow those of Smith's Table 6, are given here in the lower part of Table 2. If the genetic hypothesis is correct, then 53.8 recessives (i.e., Au(1)) are expected, and 56 are seen, a very close fit ($\chi^2 = 0.412$, $0.7 > p > 0.5$). Included in this calculation are eight families with at least one positive offspring in which the phenotype of one parent was Au(0) and in which the other parent was not tested. It was assumed in each case that the untested parent was Au(0). The probability of a positive by positive mating is about 1/100 in this population.

The results are summarized in Table 3.
Table 2. Segregation of Au(1) in families with at least one Au(1) child.

<table>
<thead>
<tr>
<th>Number of children in family</th>
<th>Number of families</th>
<th>Observed Recessives</th>
<th>Expected Recessives</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>6</td>
<td>6,000</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>5</td>
<td>6,565</td>
<td>1.110</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>8</td>
<td>6,856</td>
<td>1.960</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>8</td>
<td>12,708</td>
<td>4.692</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>6</td>
<td>7,743</td>
<td>3.246</td>
</tr>
<tr>
<td>Totals</td>
<td>24</td>
<td>42</td>
<td>40,062</td>
<td>11.008</td>
</tr>
</tbody>
</table>

\[ x^2 = 0.341 \]

\[ \frac{0.7 > p > 0.5}{x^2} \]

Table 3. Summary of calculations by Smith's method for Cebu and Bougainville families, showing variance and \( x^2 \) (1 degree of freedom).

<table>
<thead>
<tr>
<th>Location</th>
<th>Type of mating</th>
<th>No. of families</th>
<th>Observed Recessives</th>
<th>Expected Recessives</th>
<th>Variance</th>
<th>( x^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cebu</td>
<td>Au(1) X Au(0)</td>
<td>7</td>
<td>12</td>
<td>12.189</td>
<td>3.556</td>
<td>0.010</td>
</tr>
<tr>
<td>Cebu</td>
<td>Au(0) X Au(0)</td>
<td>24</td>
<td>33</td>
<td>32.705</td>
<td>8.069</td>
<td>0.010</td>
</tr>
<tr>
<td>Bougainville</td>
<td>Au(1) X Au(0)</td>
<td>24</td>
<td>42</td>
<td>40.062</td>
<td>11.008</td>
<td>0.341</td>
</tr>
<tr>
<td>Bougainville</td>
<td>Au(0) X Au(0)</td>
<td>41</td>
<td>56</td>
<td>53.815</td>
<td>11.593</td>
<td>0.412</td>
</tr>
</tbody>
</table>

The segregation analysis method recently introduced by Li and Mantel was also used to test the genetic hypothesis in the Au(0) X Au(0) families. In this, the total number of children \( t \) who are the issue of Au(0) X Au(0) matings, the number of recessives \( r \) (i.e., Au(1)) among these, and the number of children \( j \) who are the only recessive offspring in the family are determined. The proportion

\[ p' = \frac{r - j}{t - j} \]

is 0.250 for an “ideal” recessive trait. Li and Mantel give the rationale for this calculation and show that it compares favorably with other methods of segregation analyses. The results of these calculations are shown in Table 4, in which they are compared to the calculations for the Cebu data. For the Cebu data the value of \( p' \) is 0.2461 (±0.0585), a close fit, and for Bougainville the value is 0.2527 ± 0.0452, also a nice fit. Both of these results compare favorably to the values used as an example by Li and Mantel.
Table 4. Summary of calculations by Li and Mantel’s method for Cebu and Bougainville
$Au(0) \times Au(0)$ families.

<table>
<thead>
<tr>
<th></th>
<th>$t$</th>
<th>$j$</th>
<th>$r$</th>
<th>$p'$</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cebu</td>
<td>85</td>
<td>20</td>
<td>36</td>
<td>0.2461</td>
<td>0.0585</td>
</tr>
<tr>
<td>Bougainville</td>
<td>124</td>
<td>33</td>
<td>56</td>
<td>0.2527</td>
<td>0.0561</td>
</tr>
</tbody>
</table>

These segregation analyses taken together with the previous Cebu study strongly support the hypothesis that the trait follows simple Mendelian segregation in at least these two populations.

If the distributions in the families were due to simple infection with no genetic effect, then it would be expected that the frequency of Australia antigen in the genetically unrelated spouses of $Au(1)$ individuals would be about the same as the frequency in the genetically related children. There were 33 families in which both parents were tested and at least one was $Au(1)$. There were 27 $Au(1)$ among the 97 offspring, but none of the mates were positive, and this difference is significant ($0.01 > p > 0.001$).

Although there may be some nongenetic explanation for this distribution, as suggested in our previous paper, the close correspondence of the observed to the expected numbers of recessives in two quite different communities (Cebu and Bougainville) adds weight to the validity of the genetic hypothesis.

The data summarized in the introduction indicate that Australia antigen may also behave as an infectious agent. These findings are compatible with the explanation that there is an inherited susceptibility to chronic infection with Australia antigen mediated by the $Au^1$ gene. In populations where the $Au^1$ gene is relatively common and where the infectious agent is also common, then all those individuals susceptible to the agent would become infected and the segregation in the families would appear to follow a pattern of autosomal recessive inheritance.

By analogy with other examples of inherited susceptibility to infection, there are probably factors such as age, sex, state of nutrition, etc. which also affect susceptibility and resistance. Under appropriate circumstances an individual without the inherited susceptibility factors could become infected. Most patients in the United States who contract acute viral hepatitis (usually post-transfusion hepatitis) may have the antigen transiently, but it is only rarely chronic, since they are not genetically susceptible to the chronic infection. In these patients, the presence of the antigen is usually associated with evidence of clinical hepatitis and/or highly elevated serum glutamic pyruvate transaminase levels. In individuals with chronic Australia antigen, such as the large numbers of apparently normal individuals in Asia and Oceania, the effect on the liver is much less striking and may be reflected only in minor elevations of serum glutamic pyruvate transaminase, or in no changes at all. A symbiotic accommodation of the organism with the host could have developed in these individuals over the course of many generations, and even though they become infected more readily, they do not develop severe symptoms of the disease. This may represent a protective value of the $Au^1$ gene and could be associated
with the development of what appears to be a genetic polymorphism in this population.\textsuperscript{30}

The data presented here are compatible with the hypothesis that there is an inherited susceptibility in humans to chronic infection with a virus of hepatitis. The susceptibility, in this analysis, appears to be controlled at a single autosomal locus, and individuals homozygous for a recessive gene termed Au\textsuperscript{3} are more likely to be chronically infected. The infection is not accompanied by any apparent illness, although people with Australia antigen are probably hepatitis carriers.

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Vieručec, A., manuscript in preparation.


RELATION OF AUSTRALIA ANTIGEN TO VIRUS OF HEPATITIS

Dr. Blumberg


Australia antigen—Au(1)—was first identified in the serum of an Australian aborigine, using an antiserum from a hemophiliac who had received a large number of transfusions. It soon became apparent that Au(1) was closely related to hepatitis virus or that it might be located directly on such a virus.

The evidence in favor of this interpretation can be summarized as follows:

1) Au(1) is uncommon among normal persons in the U.S. (0.1%) but is frequent among patients with acute viral hepatitis (approximately 40% of those with infectious hepatitis, and 60% of those with post-transfusion hepatitis). Au(1) is not found in patients with other liver diseases.

2) Au(1) occurs in about 30 percent of institutionalized patients with Down’s syndrome, in whom it is associated with chronic (anicteric) hepatitis as shown by liver biopsy and elevated SGPT levels.

3) The antigen can be isolated by density gradient sedimentation. Under the electron microscope, Bayer et al. observed that it is a particle with a diameter of 200 A and superficial knob-like “subunits” with a diameter about 30 A. Addition of specific anti-

Au(1) antiserum results in the agglutination of these particles.

4) Fluorescein-labeled antibody against Au(1) has been produced by Millman et al. In general, patients with hepatitis and Au(1) in their blood manifest striking fluorescent granules in or on the nuclei of their hepatic cells when the fluorescent anti-Au(1) is added, whereas the liver cells of patients without hepatitis or Au(1) do not have such granules.

5) Following transfusion with blood from donors with Australia antigen, recipients may develop clinical hepatitis and Australia antigen in their sera. These changes have been observed in patients in the United States, and by Okochi and Murakami in Japan. Some patients, following transfusion, may develop antibodies against Au(1).

6) In epidemiologic studies, Au(1) behaves as if it were infectious. The agent has been found in up to 30 percent of patients with Down’s syndrome in large institutions, but rarely among patients with this syndrome in small institutions, and not at all among outpatients.

The detection of Australia antigen by the “Au test” is useful in the early diagnosis of acute hepatitis and for the detection of hepatitis in blood donors. It is now being used for this purpose in hospitals in Philadelphia.

Au(1) is found transiently in acute hepatitis, but is persistent in certain chronic diseases, notably Down’s syndrome and leukemia, and is also found in some apparently normal persons who live in the tropics.

References

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VIRAL HEPATITIS, POSTNECROTIC CIRRHOSIS, AND HEPATOCELLULAR CARCINOMA

Sir,—Primary hepatoma most commonly arises in patients with postnecrotic cirrhosis ("post-hepatitic cirrhosis") and this suggests the possibility of a link between the causative agents of primary hepatocellular carcinoma and viral hepatitis. In order to test this possible relationship, a long-term prospective study will be necessary. Two discoveries make such a study possible. The first is Australia antigen (Au[1]), detected by double diffusion in agar with specific antiserum. This particulate, virus-like antigen, originally described by Blumberg, occurs transiently in the serum of patients with acute hepatitis and chronically in the serum and liver-cell nuclei of patients with persistent anicteric hepatitis. Some of the patients with anicteric hepatitis have been shown, by liver biopsy, to have early changes of postnecrotic cirrhosis. The second discovery was that α-fetoprotein (AFP), a normal α-globulin of fetal serum, reappears in the serum of certain adults with primary hepatocellular carcinoma, and that the test for AFP (by double well diffusion using specific antiserum) may be useful in the diagnosis of primary hepatoma.

In order to obtain preliminary information on this problem, we have completed a retrospective study on the occurrence of Au(1) in the serum of 65 patients with the proven diagnosis of primary hepatocellular carcinoma, and in the serum of patients with other liver diseases. The results are detailed in the accompanying table. The frequency of Au(1) in the sera of patients with hepatocellular carcinoma does not differ from the frequency in persons in a similar population not having hepatocellular carcinoma.

This study does not support the hypothesis that the agent causing viral hepatitis (which is presumably identified by Australia antigen) is also an aetiological factor in primary hepatoma. These findings, however, do not rule out the possibility. Au(1) might be present in the serum early in the course of disease but absent at a later time. Alternatively, Au(1) may be present only in the nuclei of liver or other cells, and not in the peripheral blood. If this is true, then the antigen might be detected using the recently described Au(1) fluorescent-antibody technique. Using this technique Au(1) has been detected in liver-cell nuclei of patients with viral hepatitis, as well as in a non-transfused patient with postnecrotic cirrhosis who did not have Au(1) in the serum.

This question could be answered by a prospective study of patients in a group with a high-risk for the development of hepatocellular carcinoma. This study should include liver biopsy and fluorescent-antibody staining of cells for Au(1), with histopathological correlation.

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J. BRUCE SMITH
BARUCH S. BLUMBERG.
THE LOCALIZATION OF AUSTRALIA ANTIGEN BY IMMUNOFLUORESCENCE

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Since the discovery of Australia antigen, Au(I), by Blumberg et al. in 1965 (1) the association of this antigen with viral hepatitis has been well established (2-4). Au(I) is present in 58% of patients with posttransfusion hepatitis and in 38% of patients with infectious hepatitis (5). When patients are tested serially after blood transfusion, the incidence of Au(I) was found to be 74% (6). In most acute cases, the antigen appears in the blood early in the disease and disappears within 2-3 wk as clinical improvement occurs.

In addition to the epidemiologic association, there are several findings which provide strong support for the hypothesis that Au(I) is an antigenic determinant of a virus capable of causing hepatitis. (a) Purified Au(I), isolated from serum of patients with hepatitis, consists of virus-like particles, 200 Å in diameter, which are agglutinated by specific anti-Au(I) antiserum (7). (b) Patients who have received blood containing Au(I) have developed hepatitis with Au(I) in their blood or remained healthy and developed antibody to Au(I) (4). (c) With the use of a specific fluorescein-conjugated antibody to Au(I) we were able to demonstrate fluorescent granules in or on the surface of nuclei of cells from liver tissue in four patients with hepatitis and Au(I) in their serum. Five control patients who had neither hepatitis nor serum Au(I) did not have fluorescent granules (8).

The purpose of the present study is to determine whether the fluorescent particles in liver tissue are associated with Au(I) in the blood and whether the particles are present in tissues other than liver. This study involves 61 liver biopsy specimens, 25 specimens of other human tissues, cells from 8 samples of bile or duodenal drainage and peripheral lymphocyte cultures from 8 patients. This study confirms our previous results and establishes: (a) that the fluorescent particles in the cells are related to Au(I) in the serum and to the diagnosis of hepatitis. (b) The fluorescent particles are present in tissues other than liver in some patients.

* This work was supported in part by United States Public Health Service Grants CA-08069, CA-06927, FR-05539, FR-0040, and 5 MO1 RR40 from the National Institutes of Health, the Brian William Donovan Memorial Fund, and by an appropriation from the Commonwealth of Pennsylvania. V.C. was in receipt of a Damon Runyon Cancer Research Fellowship.
Materials and Methods

Collection of Specimens.—61 liver biopsy specimens were obtained from 58 patients in hospitals in the Philadelphia area and one from elsewhere. In 57 cases needle biopsies were performed using a Menghini needle. Two liver specimens were obtained at surgery and two at autopsy. Two patients had a second biopsy performed 4–6 wk after the initial biopsy. The second specimen in each case was treated and recorded as a separate specimen for statistical analysis. Eight bone marrow specimens were obtained by needle aspiration of the sternum. The other tissues were obtained from organs removed at surgery for therapeutic reasons. Buffy coat smears and peripheral lymphocytes were obtained from patients in our Clinical Research Unit. Seminal fluid was obtained from one patient with acute hepatitis who also had Au(I) in his serum. A blood sample was obtained from each patient at the time of liver biopsy or collection of other specimens.

One patient in our Clinical Research Unit was studied in detail and will be reported elsewhere (9). He was a 21 year old male (D.M.) with Down's syndrome and chronic hepatitis and Au(I) in his blood who later developed acute myelogenous leukemia. A bone marrow specimen was obtained 3 wk prior to death, and immediately after death specimens of liver, spleen, mesentery, and testis were taken.

Preparation of Tissues for Examination.—Each tissue specimen was placed in a small volume of McCoy’s 5a tissue culture medium (Grand Island Biological Co., Grand Island, N.Y.) containing 30% newborn calf serum (Hyland Laboratories, Los Angeles, Calif.) and was minced to produce a cell suspension. A drop of the cell suspension was placed on a clean slide, air-dried and fixed in acetone at room temperature for 10 min. At the same time, a piece of tissue was placed in formalin and processed for routine histologic examination by the clinical pathology departments of the respective hospitals. The bone marrow specimens were prepared by placing a drop of the marrow directly on a glass slide, air-drying and fixing as above. Cells were obtained from bile, duodenal drainage, and seminal fluid by centrifugation for 10 min in a clinical centrifuge, washing the cell pellet in Earle’s balanced salt solution (Grand Island Biological Co.), resuspending in McCoy’s medium, and preparing slides as above. Buffy coat smears were made from peripheral blood and fixed in acetone for 10 min. Peripheral lymphocyte cultures were prepared by the method of Hungerford (10) with the omission of colchicine.

Preparation of Reagent and Staining of Slides.—Fluorescein-conjugated rabbit anti-Au(I) antiserum was prepared and direct fluorescent staining of slides was performed by the methods previously published (8). Replicate slides were stained with fluorescein-conjugated goat vs. human antiserum and goat-vs.-rabbit antiserum to test the specificity of the reaction. Indirect staining was performed by flooding the slides with unconjugated rabbit anti-Au(I) antiserum, washing in buffered saline, and then applying fluorescein-labeled goat-vs.-rabbit anti-globulin antiserum. Control slides for the indirect test were treated first with normal rabbit serum in place of the anti-Au(I) antiserum before the application of conjugated goat-vs.-rabbit anti-globulin antiserum.

1 These include: The American Oncologic Hospital, Children’s Hospital of Philadelphia, the Hospital of the University of Pennsylvania, Jeane’s Hospital, Jefferson Medical College Hospital, Philadelphia General Hospital, Pennsylvania Hospital, Temple University Hospital, the Philadelphia Veterans Administration Hospital, and Woman’s Medical College Hospital. One case was obtained from the Strong Memorial Hospital, Rochester, New York.

2 McCoy’s medium alone or buffered saline, pH 7.4, were used in some cases and produced similar results.

3 We have tried direct tissue impressions on many liver specimens in the past and found that they were unsatisfactory.
Testing of Serum.—Serum from each patient was tested for the presence of Au(I) by double diffusion in agar gel (Ouchterlony method), using a panel of anti-Au(I) antisera consisting of rabbit, mouse, and human antisera (11).

Examination of Slides.—Slides were examined for fluorescence in dark field using a Zeiss microscope with the HBO 200 watt illuminator, BG 12 exciter filter, and 53 barrier filter. All slides were read by one of us (V.C.) without knowledge of the histologic diagnosis or the serum results for Au(I). The reproducibility of results was established by examining duplicate slides from the same patients and by reading the same slides on different days. To test the accuracy of reading, 10 slides were chosen at random and coded. They were read by V.C. and I.M. independently. The correlation between the two observers was 100%.

RESULTS

I. Liver Biopsies

Appearance of Fluorescence.—When tested by the direct method, the liver biopsies could be divided into two groups on the basis of fluorescence. In the first group, which we called negative, the cytoplasm of the cells usually appeared dull orange-brown almost identical to the autofluorescence seen in unstained preparations. The nuclei of cells in this group always appeared completely dark (Fig. 1). In the second group, which we called positive, we could detect apple-green fluorescence in, or on the surface of, the nucleus and occasionally in the cytoplasm. The nuclear fluorescence appeared in three different forms: (a) discrete granules varying in size and number, but usually averaging one to three per cell (Fig. 2); (b) diffuse finely granular fluorescence of the entire nucleus (Fig. 3a); (c) fluorescence of the nuclear rim (Fig. 3b). The first type was the most common, but all three types could be seen in the same specimen in some cases. In the positive cases, most of the cells on the slide showed some form of fluorescence. In some cases discrete fluorescent granules were seen in the cytoplasm as well as in the nucleus. Control slides stained with fluorescein-conjugated goat-vs.-human or goat-vs.-rabbit antisera, not expected to react with Au(I), showed neither intranuclear nor discrete cytoplasmic fluorescence. Occasionally we observed a pale green diffuse cytoplasmic fluorescence in both positive and negative slides as well as in some control slides. This was considered nonspecific.

The indirect method of staining demonstrated fluorescent granules only in those preparations that were positive by the direct stain. However, the granules were located to a large extent in the cytoplasm and intranuclear particles were few. In some cases the nucleus was disrupted and fluorescent granules were dispersed in the cytoplasm (Fig. 4a, b). We interpreted this to indicate that the particles had been dislodged from the nucleus by the prolonged washing procedure. Therefore, we confined our observations to the direct method.

Classification of Patients.—Table I lists the patients on whom liver biopsies were performed according to their clinical diagnosis, the histologic diagnosis made on the liver specimen, the presence of Au(I) in the serum, and the
presence of fluorescence. 33 patients had a clinical and histologic diagnosis of viral hepatitis including acute infectious, posttransfusion, chronic recurrent, and

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>Histologic diagnosis</th>
<th>Number of patients</th>
<th>Number with Au(l)</th>
<th>Number with positive fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infectious hepatitis</td>
<td>Viral hepatitis</td>
<td>9</td>
<td>5</td>
<td>8</td>
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<tr>
<td>Posttransfusion hepatitis</td>
<td>Viral hepatitis</td>
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<tr>
<td>Hepatitis in drug users (exposure to needles)</td>
<td>Viral hepatitis</td>
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<tr>
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<td>2</td>
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<tr>
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<td>Chronic active hepatitis</td>
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<td>Mild inflammatory changes to</td>
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<td>10</td>
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<td>early postnecrotic cirrhosis</td>
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<tr>
<td>Down's syndrome, and leukemia</td>
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<tr>
<td>Postnecrotic cirrhosis</td>
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<td>0</td>
<td>1</td>
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<tr>
<td>Mental retardation (institutionalized)</td>
<td>Normal liver</td>
<td>3</td>
<td>1</td>
<td>3</td>
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<td>Biliary cirrhosis</td>
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<td>0</td>
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<tr>
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<td>1</td>
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<td>0</td>
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<tr>
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<td>Laennec's cirrhosis</td>
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<td>0</td>
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<tr>
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<td>Hemosiderosis and intravascular sicking</td>
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<td>0</td>
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<tr>
<td>Totals</td>
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<td>61</td>
<td>26</td>
<td>41</td>
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chronic anicteric hepatitis. Two patients were chronic alcoholics and drug users who had been exposed to needles. One patient had postnecrotic cirrhosis which was presumed to have resulted from previous viral hepatitis. One pa-
tient with chronic renal disease had received many blood transfusions. The 24 remaining patients had a variety of other diagnoses.

**Relationship between Fluorescence and Serum Au(1).**—Table II A gives the comparison between the occurrence of fluorescence in the liver cells and the presence of Au(1) in the serum. There were 26 patients who had Au(1) in their blood. 24 had a diagnosis of viral hepatitis, one was a patient with chronic renal disease who had received many blood transfusions, and the other was an institutionalized mentally retarded patient with both Au(1) and antibody to Au(1) in his serum, but a histologically normal liver. All 26 patients with

<table>
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<th>Fluorescence</th>
<th>Number of patients with serum Au(1)</th>
<th>Total</th>
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<td>41</td>
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<tr>
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<td>20</td>
</tr>
<tr>
<td>Totals</td>
<td>35</td>
<td>61</td>
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"Chi square = 19.58; P value <0.001"

**Relationship between Fluorescence and Hepatitis.**—Table II B gives the comparison between the occurrence of fluorescence and the histologic diagnosis of viral hepatitis. 30 out of 33 patients with evidence of viral hepatitis (91%)
showed nuclear fluorescence. The three patients with hepatitis who did not have fluorescence were in the recovery phase of their illness and did not have Au(I) in their serum.

Results of Repeat Biopsies.—Both patients on whom a repeat biopsy was performed had Au(I) in their serum and acute hepatitis at the time of the first biopsy. In both cases fluorescent granules were present in the first specimen. In one patient fluorescence was not seen on the second specimen, taken 4 wk after the first biopsy, coincident with clinical improvement and disappearance of Au(I) from the serum. The other patient had a more prolonged illness with persistence of Au(I) in his blood for at least 3 months. Fluorescent granules were still present in his second biopsy specimen, obtained 6 wk after the initial biopsy.

II. Tissues Other Than Liver

Table III lists the tissues and cells studied for fluorescence. All the tissues studied from D.M. (the patient with Down’s syndrome and acute myelogenous leukemia), taken immediately after death, showed fluorescence. The fluorescence in the liver, testis, and mesenteric connective tissue is similar to that found in the liver preparation from patients with hepatitis in that the three patterns of fluorescence were found in all these tissues (Fig. 5a, b, c). In the spleen, however, only a small percentage of the cells showed fluorescence which
was mostly in the cytoplasm. The bone marrow specimen, obtained 3 wk prior to death and consisting almost entirely of myeloblasts, did not have fluorescence (Fig. 5d).

Two of eight bone marrow specimens from patients with acute or chronic hepatitis showed fluorescence confined to the cytoplasm of the cells. These were from a patient with acute infectious hepatitis with Au(I) in the blood, and from a mentally retarded institutionalized patient who had both Au(I) and antibody to Au(I). In both of these cases only a small percentage of the cells on the slide were involved.

None of the other tissues studied including buffy coat smears, cells obtained from bile, duodenal drainage, and seminal fluid showed fluorescence. Peripheral lymphocyte cultures in six patients with chronic hepatitis and Au(I) in their serum also were negative.

**DISCUSSION**

By means of the fluorescent antibody technique, using a specific anti-Au(I) antiserum, we have demonstrated fluorescent particles mainly in the nuclei and occasionally in the cytoplasm of cells from liver biopsies. Fluorescent particles were present in 100% of patients who had Au(I) in their serum. Fluorescent granules occurred in 15 patients who did not have Au(I) in their blood. Six of these had definite clinical and histologic evidence of acute or chronic hepatitis, and 5 others probably had been exposed to viral hepatitis (Table I). We interpret these results to indicate that the fluorescence test is more sensitive in detecting Au(I) than is the precipitin test. An alternative explanation is that in some patients there is little or no cellular necrosis and consequently little or no antigen is released into the peripheral blood; however, it can be detected in the cells.

The finding of fluorescent granules in one patient with biliary atresia and another patient with biliary cirrhosis (Table I) raises the question of possible involvement by viral hepatitis in the pathogenesis of these diseases of the biliary system. Biliary atresia as an end result of neonatal hepatitis has been discussed by Alpert et al. in their study of patients with trisomy 17–18 (12).

The presence of three forms of fluorescence indicates that Au(I) is not restricted to one location within the nucleus. In most cases the fluorescence appeared as one to three small masses within the nucleus corresponding morphologically to the site of the nucleoli. The finding of fluorescence in the region of or in the nucleolus is interesting in view of the studies of Bearcroft and Peachey (13, 14). Using cytological and cytochemical methods and electron microscopy they found that there was swelling of the nucleolus and an increase in nucleolar ribonucleoprotein early in viral hepatitis. They postulated that these changes may be concerned with viral synthesis. The three forms of fluorescence which we observed could indicate stages of viral replication within the nucleolus,
proceeding to the dispersal of granules throughout the nucleus, attachment to the nuclear membrane, and eventual appearance in the cytoplasm.

The appearance of fluorescence in a few cells in two bone marrow specimens could be explained on the basis of phagocytosis of circulating antigen. The widespread presence of fluorescent particles in the tissues of D.M. may represent either massive invasion by virus in a patient who was critically ill and debilitated or a viral proliferation which was directly related to the leukemic process.

We believe that the fluorescent antibody used in this study specifically identifies Au(I) for the following reasons. (a) The immunologic specificity of the antisera was confirmed by immunodiffusion and immunoelectrophoretic analysis (8). (b) Fluorescein conjugates of antisera which did not contain antibody to Au(I) failed to produce fluorescence in the positive liver preparations. (c) The association between fluorescent particles in the liver and the presence of Au(I) in the serum is highly significant \( P < 0.001 \). (d) Most of the liver specimens negative for fluorescence were from abnormal livers, suggesting that the fluorescent antibody does not detect simply a nonspecific product of cell injury.

**SUMMARY**

We have studied the localization of Australia antigen, a particulate substance associated with hepatitis, by means of the fluorescent antibody technique. Preparations were made from 61 liver biopsy specimens taken from patients with infectious hepatitis, serum hepatitis, and a variety of other diseases. When tested with fluorescein-conjugated rabbit anti-Au(I) antisera all 26 patients who had Au(I) in their serum had specific fluorescence in their liver cells. The fluorescence appeared in three forms: as discrete particles within the nucleus, diffuse fluorescence of the entire nucleus, and fluorescence of the nuclear rim. Occasionally there were also fluorescent particles in the cytoplasm. Other specimens were tested with the fluorescent antibody including a variety of human tissues, buffy coat smears, peripheral lymphocyte cultures, and cells obtained from bile and duodenal drainage. Among these specimens, fluorescence was found in the cytoplasm of a few cells in the bone marrow of two patients with hepatitis and Au(I) in their serum, and in the liver, spleen, mesentery, and testis of one patient with leukemia, chronic hepatitis, and Au(I) in his serum. We have shown that the presence of fluorescent particles in the liver cells is strongly associated with the presence of Au(I) in the serum and the diagnosis of viral hepatitis. We believe that this study adds support to the hypothesis that Australia antigen is an antigenic determinant of a virus capable of causing hepatitis.

**BIBLIOGRAPHY**


Fig. 1a and b. Cells from liver biopsy of a patient with Laënnec's cirrhosis stained with conjugated rabbit anti-Au(I) antiserum. This patient did not have Au(I) in the peripheral blood and had no clinical or histologic evidence of hepatitis. Nuclei appear dark. Cytoplasmic particles due to orange autofluorescence of lipids. This is scored as negative for fluorescence. (a) phase; (b) ultraviolet. Magnification, \( \times 1700 \).
Fig. 2a and b. Cells from liver biopsy of a patient with chronic anicteric hepatitis who had Au(I) in his peripheral blood. The biopsy is stained with conjugated rabbit anti-Au(I) antiserum, and shows intranuclear fluorescent particles. This is scored as positive fluorescence. (a) phase; (b) ultraviolet. $\times 1700$.

Fig. 3. Liver preparations from two patients with viral hepatitis who had Au(I) in their peripheral blood. The biopsy is stained with conjugated rabbit anti-Au(I) antiserum. (a) Diffuse fluorescence of entire nucleus. (b) Fluorescence of nuclear rim, as well as intranuclear particles. $\times 1350$. 

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Fig. 4. Liver preparation from a patient with viral hepatitis stained by the indirect method. (a) Stained with unconjugated rabbit anti-Au(I) antiserum. (b) Stained with unconjugated normal rabbit serum. × 1700.
Fig. 5a, b, c, and d. Tissues from D.M. (a patient with Down's syndrome, chronic anicteric hepatitis and acute myelogenous leukemia) stained with conjugated rabbit anti-Au(I) antiserum: (a) liver; (b) mesentery; (c) testis; (d) bone marrow. X 1350.
AUSTRALIA ANTIGEN (A HEPATITIS-ASSOCIATED ANTIGEN)

PURIFICATION AND PHYSICAL PROPERTIES*

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Australia antigen, Au(l), was first detected by Blumberg in 1964 in the serum of an Australian aborigine (1). Work concerning the geographic distribution, disease association, genetics, and physical and chemical characteristics of this unusual antigen has been reviewed (2). In one of the early studies, specific anti-Au(l) antisera agglutinated particles 180-210 A in diameter which were isolated from the sera of patients with viral hepatitis (3). Furthermore, antisera to the Au(l) antigen coupled with fluorescein isothiocyanate stained intranuclear granules in the liver cells of patients with hepatitis (4). This indicates that cells of the liver of patients with hepatitis contain an antigen in common with the serum particles. All this strongly suggests that Au(l) may be, or may be part of, a hepatitis virus. The association of Au(l) with acute and chronic hepatitis has already been well documented (5-15).

One of the approaches which could shed light on the question of whether Au(l) is a virus or not is to study the physical properties of purified Au(l) particles to determine whether they are consistent with those known for viruses. It is also important to ascertain whether the particles contain nucleic acids. It is generally accepted that the hepatitis virus is small, 260 A in diameter or less, and that it survives at 56°C for 30 min and freezing at −10°C to −20°C for 1 yr or longer. It is resistant to the action of enzymes, ether, and other chemical agents (16), and is present in the blood of infected individuals since hepatitis can be transferred by needle puncture. The Au(l) antigen is also found in high concentrations in the blood of individuals suffering from chronic hepatitis, and has many of the properties of the postulated hepatitis virus, as this report will show.

Materials and Methods

Source of Au(l).—D. M. was a 21 year old male with Down’s syndrome who had chemical and histological evidence of chronic hepatitis and Au(l) in his serum when first seen by us in

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1967. These findings persisted. In early 1969 he developed acute granulocytic leukemia and was admitted to our Clinical Research Unit. During the course of his treatment he was given 1.0 mCi of NaH\(^{32}\)PO\(_4\) by intravenous injection and 24 hr later 250 ml of plasma was obtained by plasmapheresis. The patient died of leukemia 6 days later.

**Assay of Au(I).**—Immunodiffusion assays were performed by the Ouchterlony method using the modifications of Blumberg and Riddell (17). Immunoelectrophoresis was performed on 3½ x 4½ inch photographic plates, using 1.1% agarose dissolved in sodium veronal buffer, pH 8.2, ionic strength 0.038.

**Electron Microscopy.**—Samples containing Au(I) were dialyzed for 4-10 hr against distilled water at 2-5°C. For negative staining a loopful of material was placed on a carbon-formvar-coated copper grid to which an equal amount of staining solution was added. The excess fluid was immediately blotted with filter paper and the grid dried in the vacuum of the microscope. For negative staining 2% uranyl acetate (w/v) in water or 4% silicotungstate (w/v) in solution adjusted to pH 7 with 1 N NaOH was employed. Each technique gave equivalent results. The micrographs were taken with a modified Siemens Elmiskop I (Siemens America, New York) electron microscope, using double condenser, 80 kv acceleration voltage, and 50 μ objective apertures at magnifications of 40,000-80,000.

**Counting.**—Radioactivity was determined by recording the Čerenkov radiation emitted by \(^{32}\)P in a conventional liquid scintillation counter as described by Haberer (18). In preliminary experiments it was shown that the presence of 1 M sucrose, 4 M cesium chloride, and a variety of other inorganic salts did not diminish the amount of radioactivity detected. Since the radioactivity determination was carried out at 0º-2ºC, without the addition of any scintillation solution, samples up to 20 μl were completely recoverable for subsequent purification.

**Resistance of Au(I).**—(a) Enzymes: A serum fraction containing Au(I) was partially purified by the method of Alter and Blumberg (19) and standardized to a concentration which produced a sharp precipitin line when assayed by the immunodiffusion technique. 25-μl portions of this fraction were allowed to react with a series of enzymes under optimal conditions as described by the supplier (Worthington Biochemical Corporation, Freehold, N. J.) of all but one of the enzymes. These enzymes included trypsin, wheat germ lipase, neuraminidase, alpha amyrase, phosphohydrolase C, ribonuclease, and deoxyribonuclease. In addition, pronase (Calbiochem Corporation, Los Angeles, Calif.), was dissolved in distilled water in a concentration of 2 mg/ml and 5 μl of this enzyme solution was allowed to react with 25 μl of Au(I) fraction. All enzyme reactions were carried out at 37°C for 1 hr.

(b) Other agents: The effect of elevated temperatures was determined by incubating Au(I) in 0.05 M tris(hydroxymethyl)aminomethane (Tris) maleate buffer, pH 7.5, at 56º, 85º, and 100ºC for 1 hr. A similar solution containing Au(I) was exposed to ether and chloroform for 1 hr at room temperature. These solvents were removed by evaporation before immunoreactivity was determined.

**Chemical Methods.**—Deoxyribonucleic acid was assayed by the method of Dische (20) and the fluorometric method of Kissane and Robins (21). Ribonucleic acid was determined by the method of Allkaum and Umbreit (22) and protein by the method of Lowry et al. (23).

**RESULTS**

Preliminary results indicated that treatment with the enzymes did not affect the immunoreactivity or structure of Au(I). An isolation procedure was then devised in which enzymatic digestion was used to degrade other constituents without apparently altering Au(I).

**Purification of Au(I).**—Step 1. Centrifugation: 100 ml of plasma was clarified at 0º-2ºC for 1 hr at 2000 g. The supernatant was re-
centrifuged at 300,000 g for 18 hr. Au(1) was detected only in the pellet; this was dispersed in 10 ml of 0.15 M NaCl.

**Step 2. Enzyme treatment:** To the above suspension were added 4 mg of amylase, 0.4 mg of lipase, 0.2 mg of neuraminidase and 0.2 mmoles of Tris-HCl, pH 7.5. The total volume (11.4 ml) was incubated for 15 min at 37°C. Thereafter 0.6 ml of a solution containing 0.1 mmoles of CaCl₂, 4.0 mg of trypsin, and 0.1 mg of phospholipase C was added. The mixture then gelled. 2 ml of pronase (10 mg/ml) was added and the mixture incubated for 1 hr. The gel disappeared and an additional 2 ml of pronase was added. The incubation was continued for an additional hour and then the mixture was cooled in an ice bath.

![Fig. 1. Gel filtration. 5 ml of the enzyme-treated suspension was placed on a 650 x 32 mm column of Sephadex G-200 gel which had previously been equilibrated with 0.85% NaCl. Elution was with the same solution and 5 ml fractions were collected. All recovered Au(1) material eluted with the void volume (peak I).](image)

**Step 3. Gel filtration:** Portions (5 ml) of the enzyme-treated suspension were filtered through Sephadex G-200. The absorption profile is shown in Fig. 1. All fractions comprising the first peak (I), which contained Au(1), were combined. Fig. 2 shows an immunoelectrophoresis pattern of each of the separation stages. These patterns were developed by adding horse anti-human serum to the troughs. There is a decrease in the number and intensity of precipitin bands following enzyme treatments and gel filtration.

**Step 4. Sucrose gradient centrifugation:** The combined fractions of peak I from step 3 were dialyzed against 0.01 M KCl and sedimented through sucrose gradients. Fig. 3 shows an analysis of the gradient for ³²P incorporation and immunoreactivity. Fractions 16–22 with major Čerenkov radioactivity and reactivity were then combined.

**Step 5. Centrifugation in cesium chloride:** Fractions 16–22 from step 4 were sedimented in cesium chloride gradients. Fig. 4 shows the resultant profile.
Fig. 2. Immunoelectrophoretic patterns of fractions at different stages of purification. Pattern 1 is starting plasma; pattern 2 is pellet after centrifugation (step 1); pattern 3 is enzyme-treated pellet suspension (step 2); pattern 4 is enzyme-treated pellet suspension passed through Sephadex G-200 (step 3). Troughs contained horse anti-human serum which does not contain anti-Au(I).

Fig. 3. Sucrose gradient profile. Sucrose gradients were prepared and sampled by the method of Martin and Ames (26). Portions (1.0 ml) of peak I in step 3 were layered over 32.5 ml linear sucrose gradients ranging from 10 to 30% by volume, in 0.01 M Tris maleate buffer pH 7.4. The gradients were centrifuged in a Spinco SW 27 rotor (Beckman Instruments, Inc., Fullerton, Calif.), at 57,000 g for 20 hr at 2–5°C. Forty-drop fractions were removed by puncturing the bottom of the tubes. Radioactivity was determined by counting each entire fraction for 100 min; background was 20.4 cpm. Immunological reactivity of each fraction was determined as detailed in Materials and Methods. The line within the radioactivity peak represents the Au(I) positive range according to immunodiffusion.
analyzed for $^{32}$P content, absorption at 280 m$\mu$, density, and reactivity with Au(1) antiserum. Immunological reactivity was found in fractions 15–21 with greatest activity in fraction 16. The relative antigenicity of each fraction was determined by twofold dilution titration. That fraction which could produce a precipitin line at the highest dilution was considered the peak fraction. The density of the peak fraction (fraction 16) is 1.21 and it falls within both the 280 m$\mu$ and Čerenkov peaks. The 280 m$\mu$ peak seen in fraction 8 (Fig. 4) is not coincident with $^{32}$P or Au(1) activity. Its absorption profile is not characteristic of nucleic acid. Fractions 15–18 were combined and after dialysis against 0.01 m KCl were used for subsequent studies.

Analysis for Immunoreactivity.—Fig. 5 shows an immunoelectrophoretic analysis of the most purified fractions (15–18) derived from step 5. There is no reactivity with horse anti-human serum (pattern 2), indicating the absence of...
serum proteins (pattern 3). In contrast, Au(I) is seen in pattern 1 (arrow), in which the purified material was reacted with human anti-Au(I). A precipitin band appears to be in the same position and has the same configuration as the band formed by unpurified Au(I) (not shown). The presence of Au(I) in the most purified fractions (step 5) indicates resistance of the antigen to the enzyme treatments and to gradient manipulations. The absence of detectable serum components in purified fractions 15–18 was confirmed by immunodiffusion test.

**Fig. 5.** Immunoelectrophoretic patterns of purified Au(I) fractions 15–18 (see Fig. 4). Pattern 1—Au(I) band (arrow) developed with anti-Au(I) in trough below. Pattern 2—Au(I) reacted with horse anti-human serum placed in trough below. Pattern 3—Whole plasma, containing Au(I), developed with horse anti-human serum in trough above.

**Extraction with Phenol or Ether.**—The small amount of $^{32}$P in the most purified fraction prior to phenol extraction does not appear to be associated with nucleic acids of high molecular weight. 2 mg of calf thymus deoxyribonucleic acid as a carrier was added to the combined fractions 15–18 of step 5. After extraction with phenol by the procedure of Thomas and Abelson (24) and centrifugation of the aqueous phase in a cesium chloride gradient, no nucleic acid as measured by $^{32}$P radioactivity was detected. It is possible that phenol did not dissociate nucleic acid from the protein. In a parallel isolation procedure in which enzyme treatment was omitted, 70% of the $^{32}$P radioactivity in the most
purified fraction was extracted with ether. There was no nucleic acid detected chemically. The sensitivities of these methods indicated that if nucleic acids were present, the concentration of DNA was less than 1% and the concentration of RNA less than 10% of the total weight of protein.

Effect of Other Agents.—Heat treatment at 85° and 100°C destroyed detectable immunological reactivity as revealed by immunodiffusion test. There was no apparent decrease in reactivity after heating Au(1) solution to 56°C for 1 hr and the reactivity was retained in the unheated control. After ether and chloroform treatments, two precipitin bands appeared where only one had been seen previously. This may indicate that these treatments uncover other antigenic groups. One of the precipitin bands of the ether-treated Au(1) fraction appeared to be different from one of the two bands of chloroform-treated Au(1) fraction, since they crossed, i.e., did not form a line of identity.

Electron Microscopy of Purified Au(1).—Fig. 6 is an electron micrograph of Au(1) present in fractions 15–18 of the cesium chloride gradient. In some of the particles a central "core" was observed. Particles were found in all those cesium chloride fractions which produced a precipitin band by immunodiffusion (Fig. 4, fractions 15–21). The greatest concentration of particles appeared to be in fraction 16. Heat (100°C), ether, or chloroform did not affect the appearance of Au(1) as seen under the electron microscope. However, the particles disap-

![Fig. 6. Negatively stained particles from purified Au(1) fractions 15–18 of the cesium chloride gradient. Magnification 300,000. Bar indicates 1000 Å.](image)
peared completely after treatment with Carnoy’s solution (3 parts ethanol:1 part glacial acetic acid) followed by pronase digestion.

DISCUSSION

Australia antigen was apparently not affected by enzyme digestion as determined by its immunological characteristics and its appearance under the electron microscope. The remarkable stability despite such treatment made it possible to remove most other human serum proteins as a preliminary step in the purification of Au(1). The absence of host-related material (i.e. serum proteins) was demonstrated by a sensitive criterion, namely, the lack of immunological reactivity with horse anti-human serum in immunoelectrophoretic and immunodiffusion analysis.

The buoyant density of purified Au(1) was 1.21. This value confirms the earlier result of Alter and Blumberg (19). This density is lower than that reported for most picorna- and parvoviruses (which are comparable in size to Au(1) particles); these range between 1.32 and 1.4 (25). The lower density of purified Au(1) could result from the absence of nucleic acid and/or the presence of lipids. An external lipid coat has not been reported in viral agents of this size range. The presence of lipids in Australia antigen has previously been postulated on the basis of the staining reactions (19). Exposure to ether and chloroform appears to uncover previously masked antigenic groups; when the treated Au(1) was tested against both human and rabbit anti-Au(1) an additional precipitin band was detected.

The morphology of the particles isolated from the cesium chloride gradient, as revealed by the electron microscope, does not differ from that already reported by Bayer et al. (3). The particles are 180–210 A in diameter and in most cases spherical in shape. They have central cores and appear to be composed of subunits. A few elongated particles with the same diameter (3) have been observed in the present material. Considering the size of these particles they could be compared to the adeno-associated (AAV), parvo-, and picornavirus groups (25). In the most purified fractions there is no evidence for the presence of significant amounts of nucleic acid. Thus, it is possible that the $^{32}P$ seen in the gradients containing Au(1) may have been associated with lipids or lipoproteins which were subsequently removed by the phenol extraction.

The tentative hypothesis is that the bulk of material identified as Australia antigen may be an incomplete virus or virus capsid associated with viral hepatitis. Studies are currently directed at isolating an infectious particle.

SUMMARY

Australia antigen [Au(1)], a particle associated with viral hepatitis, was isolated from the plasma of a patient with chronic anicteric hepatitis and leukemia who had received radioactive phosphorus.
We have found that the immunoreactivity and appearance of Au(1) in the electron microscope were not altered by treatment with enzymes including trypsin, pronase, lipase, phospholipase C, ribonuclease, deoxyribonuclease, amylase, and neuraminidase. In contrast, other serum constituents were degraded by these enzymes. Therefore, treatment of the patient’s plasma with many enzymes was exploited as an initial step for the isolation of Au(1). Subsequently, Au(1) was purified from the enzyme-treated 32P-labeled plasma by gel filtration through Sephadex G-200 and centrifugation through sucrose and cesium chloride gradients. There were no detectable human serum components in the purest fractions, as tested by immunoelectrophoresis and immunodiffusion. The density of the purified Au(1) was 1.21 in CsCl. The particle measured about 200 Å in diameter, was predominantly spherical in shape and appeared to be composed of subunits. Nucleic acids were not detected by spectrophotometric, radiochemical, and chemical analyses. Immunoreactivity of purified Au(1) was destroyed by heating for 1 hr at 85°C but was stable at 56°C. Treatment with Carney’s solution (3 parts ethanol:1 part glacial acetic acid) followed by pronase disrupted the particles as seen with the electron microscope. These findings, combined with other published information on Australia antigen and viral hepatitis, suggest that the bulk of Australia antigen in the blood of this patient is an incomplete virus or virus capsid.

BIBLIOGRAPHY


Australia Antigen and Viral Hepatitis in Drug Abusers

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Much evidence supports the hypothesis that Australia antigen (Au(l)) causes viral hepatitis. We found Au(l) in 30 of 43 drug abusers with hepatitis (69.8%), a similar frequency to posttransfusion hepatitis (56.3%), but different from infectious hepatitis (30.1%). There were no distinctive histologic features in liver biopsies from drug abusers. Two of nine drug abusers with Au(l) followed serially, developed persistent Au(l); two of 22 other Au(l) hepatitis patients had persistent Au(l). Australia antigen was found in nine of 215 "normal" addicts (4.2%), significantly different from the general population (two of 2,412), but not different from 261 nonaddicts exposed to hepatitis by multiple blood transfusions (3.8%). Acute hepatitis in drug abusers is likely primarily viral, not toxic in origin.

There is now a great deal of evidence to support the hypothesis that Australia antigen (Au(l)) is a causal agent of viral hepatitis. Persistent serum Au(l) has been found in patients with subacute and chronic active hepatitis, and in up to 20% of apparently normal people in some tropical populations. Persistent Au(l) also occurs in leukemia.

Materials and Methods

The subjects of this study are 258 habitual users of addicting drugs (ie, opiates, amphetamines, barbiturates). Users of marijuana alone were not included. Forty-three had acute hepatitis at the time they were studied, and 215 were asymptomatic and presumably healthy. There were 231 men and 27 women. The age range was from 16 to 58 years with a median of 23. The clinical diagnoses of the patients with acute hepatitis were supported by elevated serum glutamic pyruvic transaminase (SGPT) levels and abnormalities in other liver chemistry tests. Nineteen of these patients had liver biopsies performed which without exception supported the diagnosis of acute hepatitis. These patients were compared with 103 other patients with viral hepatitis who were not known drug abusers. The latter group was obtained since the series of hepatitis cases reported in our previous study, and blood was generally drawn soon after the onset of symptoms. Australia antigen commonly appears only transiently early in the course of hepatitis; this is reflected by a higher frequency of Au(l) in the more recent series. This group includes 32 patients with posttransfusion hepatitis, and 71 patients without a history of transfusions or injections, whose condition was diagnosed as infectious hepatitis. To gauge the effect of drugs on the persistence of Au(l), nine drug users with hepatitis and Au(l) in their blood were followed up for two months or longer and compared with 22 other hepatitis patients with Au(l) who do not use drugs and who were followed up for the same length of time.

A group of 261 patients at Jefferson Medical College Hospital who had received multiple blood transfusions were used as a control group for the drug addicts without clinical hepatitis. Patients with leukemia and chronic renal disease were excluded, since they are known to have a high frequency of persistent Au(l). Australia antigen was determined by immunodiffusion in agar gel by a micro-Ouchterlony technique. Determinations of SGPT were performed by the method of Wroblewski and LaDue. Liver biopsy specimens were obtained with a Menghini needle, and the sections were stained with hematoxylin-eosin and special stains. Eleven drug abusers with hepatitis and Au(l) in their blood were matched by sex and age with 11 other patients with acute hepatitis. The histologic characteristics of their liver biopsies were compared. Statistical comparisons were made by the chi-square test or the sign test.

Results

Nine of the 215 asymptomatic drug abusers (4.2%) had Au(l) in their blood. This was significantly different from the frequency previously reported in the general population in the United States (0.1%). A group of 261 multiply transfused patients who were not drug abusers (3.8%) (X² = 70.1667, P < 0.0001). However, it was not significantly different from the frequency in the 261 multiply transfused patients who were not drug abusers (3.8%) (X² = 0.0015) (Table 1). Australia antigen was found in 30 of the 43 drug abusers with hepatitis (69.8%) (Table 2). This is close to the frequency found in the 32 patients with posttransfusion hepatitis (56.3%), and is significantly
This study has demonstrated a high frequency of Au(1) in drug abusers with hepatitis in the Philadelphia community. This frequency is not significantly different from that found in patients who develop hepatitis following transfusions as reported in this and earlier studies. 6-17; it is significantly higher than that in hepatitis patients who had no transfusions or injections. These results are in agreement with a recent report by Cherubin et al in New York. 18

There are now a variety of published data of a clinical, immunological, epidemiological, and electron microscopic nature favoring the hypothesis that Au(1) is a virus, or part of a virus, which causes human hepatitis (see reference 4 for summary). Australia antigen is not associated with toxic hepatitis. 15 The high frequency of Au(1) in drug abusers with hepatitis thus favors a viral rather than a toxic etiology of hepatitis in these patients, as has been suggested by others. 20-21 The significant difference from the frequency in infectious hepatitis and the similarity to that in patients with posttransfusion hepatitis suggests that the mode of transmission is the same as in the latter. However, two drug abusers with Au(1) and hepatitis gave histories of solely oral drug intake, no transfusions or injections, but contact with other drug abusers who contracted hepatitis from needles.

There is a possibility that more than one agent may be transmitted simultaneously with the same contaminated equipment. This was suggested by a young man who developed severe acute hepatitis with Au(1) in his blood accompanied by an elevation of heterophil antibody titer and a rising EB virus antibody titer, which indicates the presence of infectious mononucleosis as well. It was considered likely that both Au(1) and the virus of infectious mononucleosis were transmitted by injection at approximately the same time. After a severe illness, his Au(1) disappeared, and the heterophil antibody titer returned to normal range.

Serial blood samples were obtained over a period of two months or longer from nine of the drug abusers differently, including mescaline, mariguana, amphetamine, dextroamphetamine, methamphetamine, chlor Diazepoxide hydrochloride (Librium), and seconal. He was admitted to the hospital with severe acute hepatitis. His SGPT level rose to 2,400 Karmen units, and his serum bilirubin level to 15 mg/100 ml. His serum was positive for Au(1) and he had a marked rise in heterophil antibody titer of the type seen in infectious mononucleosis. The antibody titer against EB virus rose during his hospitalization. There were marked changes of acute hepatitis with early fibrotic changes on liver biopsy. Fluorescent granules were seen in the nuclei of his liver cells after staining with fluorescein labeled antibody to Au(1) antibody. 14 Au(1) subsequently disappeared from his blood and his heterophil antibody titer diminished to within the normal range.

Comment

This study has demonstrated a high frequency of Au(1) in drug abusers with hepatitis in the Philadelphia community. This frequency is not significantly different from that found in patients who develop hepatitis following transfusions as reported in this and earlier studies. 6-17; it is significantly higher than that in hepatitis patients who had no transfusions or injections. These results are in agreement with a recent report by Cherubin et al in New York. 18

There are now a variety of published data of a clinical, immunological, epidemiological, and electron microscop
with hepatitis who initially had Au(I) in their serum. Two of these patients (22.2%) had persistent Au(I) in the last blood specimens 63 days and one year, respectively, following onset of the disease. This was not significantly different from the 9.1% frequency of persistence in 22 hepatitis patients who did not use addicting drugs.

We also demonstrated a high frequency of Au(I) in drug abusers without hepatitis compared with the general population. However, when these were compared with non-addicts who had been exposed to multiple blood transfusions, there was no significant difference. It does not appear that the abuse of drugs provokes persistence of Au(I) any more than that seen in nonusers of drugs; certainly, if it does, it is not striking. In other studies, persistent Au(I) has been shown to be associated with chronic hepatitis, and persistence of viral activity has been implicated in this condition. By analogy, it seems likely that those drug abusers with hepatitis in whom Au(I) persists are also destined to develop chronic hepatitis; it thus appears that the chronic disease in these patients is virus-induced and not drug-induced as has been suggested by others. The possibility of some influence of drugs has not been entirely excluded however.

Of practical importance is the known tendency of drug users to become professional blood donors to support their habit. An increased risk of posttransfusion hepatitis has been documented following transfusions of blood obtained from commercial sources, and at times can be associated with addict donors. Furthermore, a study of Au(I) in 5,927 blood donors has shown that certain high-risk donor groups may be identified by the "Au test." The current study further emphasizes the hazard of the use of sources of blood obtained from poorly screened donors.

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References


Ergasteric Hepatitis: Endemic Hepatitis Associated with Australia Antigen in a Research Laboratory

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Endemic hepatitis occurred among the personnel of a research laboratory working with human blood and tissues in the study of Australia antigen (Au(1)). All individuals were tested for serum glutamic-pyruvic transaminase (SGPT) levels and Au(1). Fifty-six were tested at monthly intervals on 5 to 32 occasions over 3 1/2 years. Eighty-three others were tested less frequently. Twenty-seven changes were found in the serums of the 56 people tested frequently enough to have their course examined. There were four cases of typical acute hepatitis with Au(1) in the serum, fifteen staff members with rises in SGPT indicative of acute anicteric hepatitis, and eight staff members with small SGPT rises. We have termed this laboratory-acquired infection “ergasteric hepatitis” from the Greek word for laboratory. Seven patients were treated with bed rest as soon as the SGPT exceeded 50 units; in six the levels promptly returned to normal. We recommend strict infectious precautions and monthly monitoring of SGPT and Au(1) in personnel exposed to human (or nonhuman primate) blood or tissues.

AUSTRALIA ANTIGEN (Au(1)) was first detected in the blood of an Australian aborigine in 1963 (1). Through a series of sequential studies, patients with Down’s syndrome were ultimately tested for Au(1) (2, 3); it was in one of these patients in 1966 that we found that Au(1) could be acquired. This patient also developed asymptomatic anicteric hepatitis. We subsequently determined that Au(1) was associated with acute and chronic forms of viral hepatitis (3-7). Since that time we have been testing the hypothesis that Australia antigen is located in a virus that causes hepatitis in many individuals infected with it. The data, reviewed in detail in several publications (8-10), support this hypothesis.

After we found the association of Au(1) with hepatitis, one of our staff (Institute for Cancer Research, Philadelphia, Pa.) developed acute hepatitis (in May 1967) accompanied by Australia antigen in her blood. Her case, which has been reported (11), led to the institution of a surveillance and control program for hepatitis in the laboratory. Despite this, several of our staff have developed signs, symptoms, and laboratory abnormalities (including the presence of Au(1)) associated with hepatitis. Many other laboratories throughout the world are now working with Australia antigen, and cases of hepatitis associated with Au(1) have occurred in their staffs.

Hepatitis has been an important laboratory-acquired infection for at least the last four decades (12). We have applied the term “ergasteric” (from Greek ergasterion, έργαστήριον, meaning workshop or laboratory) (13) to describe such laboratory spread of infection. This is analogous to other terms such as nosocomial (hospital-induced) and iatrogenic (doctor-induced) and serves to distinguish the special epidemiologic, prophylactic, and possibly therapeutic aspects of hepatitis acquired in the laboratory. In this paper we describe the ergasteric hepatitis problem in our laboratory and the surveillance program that has been designed to cope with it. We hope that our experience will help in the design of programs in other laboratories and also serve to point out the dangers of serum containing Australia antigen and of isolated Au(1). This hazard probably exists in all laboratories and facilities in which human blood and blood products are handled.
Materials and Methods

SURVEILLANCE AND PREVENTION PROGRAM

After the first case of ergasteric hepatitis was diagnosed in a staff member in May 1967, a monthly testing program was instituted. All staff members were tested for Au(1) by the micro-Ouchterlony double immunodiffusion technique (14), and the level of serum glutamic-pyruvic transaminase (SGPT) was measured by the method of Wroblewski and La Due (15). For a variety of reasons not all personnel could participate in all monthly testings, but the survey generally included most of the staff. The study represents 756 man-months of testing. In addition to the monthly testing, strict precautions were instituted in the laboratory for the handling of biologic materials that are potentially infectious (Table 1).

STAFF

During the time period reported here a total of 139 staff members were tested. These included physicians, scientists, nurses, technicians, laboratory aides, computer keypunch operators, and secretaries. There were 37 men (age 18 to 47) and 102 women (age 18 to 60). Sixty-nine of the personnel were in laboratories not closely associated with the Australia antigen program and were in contact with the material only briefly. Fifty-one of these people were tested once only; eighteen were tested twice. The other seventy staff members (with the exception of two secretaries and two keypunch operators) were in fairly close contact with Au(1) either in the laboratory, the Clinical Research Unit, or both. These seventy staff members were tested on multiple (3 to 32) occasions. Fifty-six were tested five or more times and were considered to have had enough tests to have their course examined. They were then classified into the following categories.

CLASSIFICATION OF SUBJECTS (TABLE 2, FIGURE 1)

Group A: This group consisted of individuals who developed typical acute viral hepatitis characterized by gastrointestinal or systemic symptoms with or without jaundice, or both, and a peak SGPT greater than 1,500 Karmen units/100 ml.

Group B: Individuals with SGPT rises from 30 to 200 Karmen units/100 ml, with an otherwise constant level below 30 Karmen units/100 ml, composed this group. These individuals had no symptoms, but their course was considered indicative of acute anicteric hepatitis.

Group C: Those who had a peak of SGPT above their usual level but still less than 30 Karmen units/100 ml made up the third group.

Group D: The last group comprised those whose SGPT was always less than 30 Karmen units/100 ml, with minimal variation from month to month.

Results

To determine the expected normal value of serum glutamic-pyruvic transaminase (SGPT) for the asymptomatic group of people, we calculated the mean (15 Karmen units/100 ml) and standard deviation (7.5 Karmen units/100 ml) of the value obtained for the first test for each of the 139 staff members. For this reason we consider 30 Karmen units/100 ml (2 standard deviations above the mean) to be the top normal level but still less than 30 Karmen units/100 ml, SGPT was always less than 30 Karmen units/100 ml, made up the third group.

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normal SGPT level for our laboratory personnel. The 56 individuals who were tested five or more times were classified into the categories defined above.

GROUP A

Four patients with typical acute viral hepatitis had maximum SGPT levels greater than 1,500 Karmen units/100 ml, along with clinical symptoms, during their illness. When they did not have acute hepatitis, their SGPT levels were below 30 Karmen units/100 ml and fairly constant (Figure 1A). Three were jaundiced, and the fourth was anicteric, her highest serum bilirubin reaching 1.7 mg/100 ml. All these patients had Au(1) in their serum during their acute disease. They are described in the following brief case reports.

Patient 1: A 24-year-old white female laboratory technician (11) was admitted to the Clinical Research Unit on 8 May 1967 with a chief complaint of dark urine and loss of appetite of 9 days' duration. Approximately 5 weeks before admission the patient noticed the onset of easy fatigability, with no other associated symptoms. Nine days before admission she noted the onset of dark urine and light stools and a slight decrease in appetite. The night before admission she had had a profuse sweat that soaked the sheets of her bed. The patient was exposed daily to serum samples, including sera from hepatitis patients with Au(1). In addition, she had participated in field trips to two large institutions for the mentally retarded, both areas of endemic hepatitis. As part of her work she had handled isolated fractions of Au(1) and, although advised to wear gloves when handling these materials, had not done so. The day before admission the patient had had her serum tested for SGPT; it was 1,400 Karmen units/100 ml. She tested her own serum for Au(1) and found that it was positive. She knew of no breaks in the skin, and her only injections were a tine (tuberculin) test 6 months before admission and an influenza immunization 1 year before admission. Because of the dark urine she visited a urologist, who found bacteria in her urine. He treated her with sulfisoxazole (Gantrisin®) for 1 week before admission. Physical examination was unremarkable, without detectable jaundice or a palpable liver. Her highest SGPT was 1,950 Karmen units/100 ml. Other liver function studies were only slightly abnormal: serum bilirubin, no higher than 1.7 mg/100 ml; alkaline phosphatase, 5.8 Bodansky units/100 ml; and thymol turbidity, 6.8 units. The urine was never positive for urobilinogen, and thymol turbidity rose to 10.7 units. Australia antigen was present in her blood on the first 2 days of admission but was subsequently absent on repeated tests. The first several days in the hospital were marked by nausea and vomiting, requiring intravenous fluid therapy. Her course thereafter was one of steady improvement; she was discharged 30 days later.

Patient 2: A 25-year-old white female technician was admitted to the Clinical Research Unit on 22 August 1968 with a chief complaint of jaundice and abdominal pain. Two weeks before admission she was unduly fatigued and developed a distaste for cigarettes. Six days before admission she had some upper abdominal pain, general abdominal discomfort, anorexia, and constipation. One day before admission she had scleral icterus and began to feel somewhat better as the icterus developed. She had no history of injections or of any breaks in the skin in the laboratory. Past medical history included jaundice for 2 weeks at age 16 years, diagnosed as hepatitis. Her physical findings included icterus, an enlarged tender liver with a soft rounded edge, and a barely palpable spleen tip. Her serum bilirubin reached 8.4 mg/100 ml and SGPT, 1,710 units. Other liver function studies showed only minimal abnormalities. The Au(1) was present on only the first blood specimen. Because she improved rapidly she was discharged, at her own request, to bed rest at home only 8 days after admission. Since the SGPT did not continue to fall, she was readmitted on 24 September 1968 and remained at bed rest in the hospital until her SGPT was less than 50 Karmen units/100 ml. She was again discharged on 15 October 1968.

Patient 3: A 20-year-old white female college student employed as a technician was admitted to the Clinical Research Unit on 17 April 1969 with an intensely pruritic rash about the ankles that had developed 3 days previously. The rash was red and raised, with sharp borders. Over the ensuing 48 hours it spread to the thighs, the flexor surfaces of the forearms, and then to the abdomen, back, flanks, and the nape of the neck. She consulted her school physician, who found bilirubin in the urine and made the diagnosis of probable hepatitis. In the 24 hours before admission she had had some decreased appetite and abdominal discomfort. She noted dark urine on the morning of admission, but the color of her stools had not changed. She had not received injections or transfusions or been cut. Before this illness she never had allergies or rashes. She denied taking drugs orally or parenterally. The patient, her sister, and her brother-in-law ate raw clams during the week of 17 March while vacationing in Florida. On physical examination she had the rash described above, slight icterus, a temperature of 100.4 F, and minimal liver tenderness on deep palpation. The SGPT was 3,240 Karmen units/100 ml on admission. Her highest serum bilirubin was 6.2 mg/100 ml, and her highest alkaline phosphatase was 12.9 Bodansky units/100 ml. Thymol turbidity rose to 10.7 units. Australia antigen was present in her blood on the first 2 days of admission but was subsequently absent on repeated tests. The first several days in the hospital were marked by nausea and vomiting, requiring intravenous fluid therapy. Her course thereafter was one of steady improvement; she was discharged 30 days later.

Patient 4: A 32-year-old white male physician was admitted to the Clinical Research Unit on 20 February 1970. He had been working in the Laboratories of the Division of Clinical Research for about 1½ years and had worked extensively with purified Au(1) for the previous 5 months. On routine testing his SGPT was found to be 90 Karmen Units/100 ml on 18 February 1970, and it increased to 200 units on 19 February. He had no symptoms, but Au(1) was found in his blood. He had had a previous transient rise of his SGPT in April 1969 to 90 Karmen units/100 ml, which was attributed to a severe cellulitis. To his recollection he had not had any breaks in his skin while working in the laboratory but had performed a necropsy on a patient with Au(1) more than 6 months before admission. He
did drink coffee and smoke cigarettes in the laboratory, a possible source of oral contact with the agent. System review showed chronic sinusitis, pseudopapilledema, and a chronic, nonproductive morning cough. The patient had had an unexplained eosinophilia (10 to 35%) for at least 12 years. Four months before admission he had had a superficial melanoma excised from his back. On physical examination he had two very small spider angioma on the dorsum of his right hand, which he had had for many years. There were no other relevant findings. Shortly after admission he lost his taste for coffee. His liver became palpable and tender about 1 week after admission, and he gradually became anorexic and somnolent. His SGPT rose to a peak level of 2,300 Karmen units/100 ml. Highest serum bilirubin was 5.2 mg/100 ml total (2.7 mg/100 ml direct); highest alkaline phosphatase was 13.2 Bodansky units/100 ml; and highest thymol turbidity was 9.5 units. Other liver function studies were unremarkable. Gradual clinical improvement was accompanied by a fall in SGPT and the disappearance of Au(1) from his blood 30 days after admission. He was discharged 2 weeks later.

GROUP B

Fifteen other patients (group B) had rises in their SGPT to a level of 30 to 200 Karmen units/100 ml without other clinical signs or symptoms or abnormal physical findings (Figure 1B). No other liver function abnormalities were found, and Au(1) was not detected in their blood.

GROUP C

Those in group C (eight people) had slight SGPT elevations above their normal level but below 30 Karmen units/100 ml (Figure 1C).

GROUP D

The other 29 patients (group D) had no remarkable rises in SGPT (Figure 1D).

Discussion

Viral hepatitis may be endemic among laboratory and clinical personnel actively engaged in the study of Australia antigen. There were four typical cases of acute viral hepatitis in our laboratory over the past 3 years; all four had Au(1) detected in their serum. In three Australia antigen had been detected for only 1 or 2 days when they first became symptomatic. This observation emphasizes the necessity for testing patients as early as possible in the course of viral hepatitis. Australia antigen was not found in any of our other laboratory personnel.
Fifteen additional individuals had transaminase rises to 30 to 200 Karmen units/100 ml, which may represent episodes of acute anicteric hepatitis. However, these 15 individuals had no symptoms and probably would not have come to the attention of a physician had they not been in the monthly surveillance program. These 19 definite and suspected cases occurred among 56 people working in the laboratory for various time periods from 1967 to 1970 and whose SGPT was tested 5 to 32 times. They occurred despite the careful precautions taken in the laboratory after the initial case of hepatitis in May 1967. Eight others had lower SGPT peaks, below 30 Karmen units/100 ml, but even these were higher than their usual levels.

The mean SGPT level in 139 normal laboratory staff members who did not experience an episode of acute viral hepatitis was 15 Karmen units/100 ml. With a standard deviation of 7.5 units, the top normal level should be about 30 units, rather than the 40-unit level used in most clinical laboratories. Most individuals had SGPT levels consistently between 8 and 15 Karmen units/100 ml, and any significant deviation from their usual level should have been suspect. It is clear, however, that if these slight but definite rises in SGPT did represent infection of the liver, they required no treatment, since those who had such rises continued their usual activity.

In regard to the staff members with transient SGPT elevations (Table 2), we suggest four alternative hypotheses: these individuals had a mild asymptomatic ergastic infection of acute viral hepatitis and recovered; they had some transient acute liver disease of other causes; they had some transient nonhepatic disorder causing a rise in SGPT; or the SGPT elevation may be within the range of normal variation. The current series of observations does not distinguish between these several hypotheses, but they should be tested.

We do not know what the variation of SGPT might be in the population at large if they were followed at monthly intervals as were the subjects of this study. The agent responsible for this disease may be ubiquitous in the population, and hepatitis may be common at asymptomatic levels. This would be consistent with the differences we found among our laboratory personnel and might represent different responses of individuals to infection with the same or similar agents. The expression of clinical disease seen in group A (four cases of acute icteric hepatitis) would thus be the result of the specific host response to an environmental agent. Other hosts might respond by presenting only minimal evidence of acute disease. Still others may permit the agent to remain in them for long periods with or without causing chronic disease. We have presented evidence (16, 17) that susceptibility to persistence of Australia antigen is inherited as a simple autosomal recessive trait. It is reasonable to consider that the capacity of the host to interact in other ways with an infectious agent would also be inherited.

Whenever we found an increase in SGPT above 50 Karmen units/100 ml in a staff member, bed rest was instituted. Six individuals so treated showed a prompt return of their SGPT to lower levels, and eventually it returned to their usual constant range. In one individual (Patient 4) progression to typical acute viral hepatitis occurred. He did not develop clinical symptoms until 10 days after his first SGPT elevation was detected, when it reached the level of 1,250 Karmen units/100 ml. Australia antigen was also detected 10 days before symptoms developed; it persisted for 4 weeks. The other three people with acute hepatitis presented at a time other than monthly testing periods with symptoms suggesting the disease. Australia antigen was detected in these three patients for only 1 or 2 days after admission, then disappeared. None of the four patients recalled any specific parenteral exposure; infection occurred either through an occult break in the skin or via another route, probably oral.

It is difficult to say whether bed rest was useful in those people whose SGPT levels came down promptly and who did not develop acute hepatitis, or whether it would have returned to normal even if they had maintained their usual activity. Furthermore, Australia antigen was not found in the blood of any of these six individuals. Previous studies have indicated that strict bed rest is of little value in the treatment of clinical acute hepatitis with jaundice (18). Strenuous exertion, however, may be harmful in the preicteric stage (19). It might now be of interest to study a group of people at high risk for developing

Table 2. Classification of 56 Laboratory and Clinical Research Staff Members by Serum Glutamic-Pyruvic Transaminase (SGPT) Levels*  

<table>
<thead>
<tr>
<th>Classification</th>
<th>Symptoms</th>
<th>Peak SGPT</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical acute viral hepatitis</td>
<td>Yes</td>
<td>&gt;1,500</td>
<td>4</td>
</tr>
<tr>
<td>Suspicious acute anicteric hepatitis</td>
<td>No</td>
<td>30-200</td>
<td>6</td>
</tr>
<tr>
<td>Minimal SGPT rise</td>
<td>No</td>
<td>&lt;30</td>
<td>8</td>
</tr>
<tr>
<td>Low SGPT, minimal variation</td>
<td>No</td>
<td>&lt;30</td>
<td>29</td>
</tr>
</tbody>
</table>

* Tested at least five times.
acute hepatitis, test them at regular intervals for Australia antigen, and evaluate the importance of early bed rest, before the development of clinical symptoms. This technique of very early detection (as in Patient 4) might also be of value in testing the usefulness of other methods of therapy (gamma globulin, steroids, antiviral agents, and so forth). Previously there has not been a tool for evaluating treatment in the earliest stages of the disease; the test for Australia antigen now makes such a study feasible.

We are not aware of any other studies in which normal people who have a high risk of developing hepatitis have been followed at frequent intervals for 3 or more years, as in the present study. Hence, we do not know if our experience with the disease is any worse or any better than that of other laboratories where large quantities of human serum are handled. Nevertheless, on the basis of our experience, all human serum or other tissues should be regarded as contaminated to help protect the laboratory personnel against infection. The rules instituted in our laboratory (Table 1) may help in such a program. The personnel of research laboratories working with Australia antigen or any human blood or tissue should be monitored for Au(1) and SGPT levels. It would also be valuable to determine the usefulness of this monitoring program for other potentially high risk groups, including personnel of clinical laboratories, pathology laboratories and morgues, blood banks and blood donor stations, renal dialysis units, centers for treatment of drug abuse, and primate colonies. In this way the diagnosis can be made as early as possible, and prompt treatment can be instituted.

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THE NATURE OF AUSTRALIA ANTIGEN AND ITS RELATION TO ANTIGEN-ANTIBODY COMPLEX FORMATION*

BY BARUCH S. BLUMBERG, IRVING MILLMAN, ALTON I. SUTNICK, AND W. THOMAS LONDON

(From The Institute for Cancer Research, Philadelphia, Pennsylvania 19111)

Studies on Australia antigen-antibody complexes have just begun. In our first electron microscope study of Australia antigen (1), complexes were formed upon the addition of antibody to the isolated antigen on the grid. More extensive electron microscope studies of apparent complexes have also been described (2). Shulman and his colleagues (3) suggested that anticomplementary activity in sera may reflect the presence of Australia antigen-antibody complexes. Millman et al. (4) have directly demonstrated that antigen may be released from antibody by centrifugation of appropriate serum; high and low avidity complexes have been described in this manner. The role of Australia antigen-antibody complexes in periarteritis nodosa (5, 6) has been explored and this will be discussed later in this symposium. Hopefully, more extensive information on the distribution of complexes in healthy and sick people will be forthcoming soon. The radioimmunoassay method developed in our laboratory by Drs. Coller and Millman (7, 8) appears to detect antibody and Au(1) complex in the serum even in low concentrations, and this may help in the elucidation of this problem.

Before we can understand the nature of antigen-antibody complexes we must know more about Australia antigen itself. Since 1963 (9, 10) we have collected a variety of data on this subject and have developed a concept over the past year of what may be considered to be a special kind of infectious agent. This concept states that Australia antigen has characteristics both of an infectious agent and a serum protein polymorphism. Most of the data to support this hypothesis are given elsewhere in our publications and will be referred to in the text.

Australia Antigen as an Infectious Agent.—After we found the association between Australia antigen and hepatitis in 1966 (11), we began to test the hypothesis that Australia antigen is a virus which can cause hepatitis. To do this we used the scientific strategy of independent evidence. That is, we looked to see if Australia antigen had characteristics similar to that of viruses. This could be called the “duck logic,” i.e. if a creature looks like a duck, flies like a

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duck, swims like a duck, eats like a duck, then it is more likely to be a duck than say a scarlet tanager.

The results of these studies, most of which have now been amply confirmed in laboratories other than our own, as shown in Table I. These can be summarized briefly here, but are discussed in detail elsewhere (12-14).

Australia antigen is associated with acute “viral” hepatitis, both “serum” and “infectious” (Table II) and with various forms of chronic hepatitis (Table III). Isolated Australia antigen has an appearance compatible with that of a virus particle of about 200 Å in diameter (Fig. 1) (1). The preparations may contain sausage-shaped figures and larger (400 Å) particles (1, 15). Millman and his colleagues (16, 17) have prepared fluorescent anti-Au(1) antiserum. With this they have shown that the liver cells of patients with Australia antigen in

TABLE I

Evidence that Australia Antigen is an Infectious Agent

(a) Association with acute viral hepatitis.
(b) Association with chronic hepatitis.
(c) Virus-like appearance under the electron microscope (200-Å particles).
(d) Transmission of Au(1) from man to man.
(e) Transmission and passage of partially purified Au(1) to an animal host (infant African green monkey).
(f) Localization [with fluorescent anti-Au(1)] of Au(1) in the nuclei of liver cells of patients with hepatitis and/or Au(1) in their blood.
(g) Distribution of Au(1) in institutions, disease groups, and populations is consistent with the distribution of an infectious agent.
(h) RNA identified in Au(1) particles isolated from blood.
(i) Apparent replication of Australia antigen in tissue cultures of human liver cells.

their peripheral blood and or hepatitis have, in general, fluorescent granules in their nuclei. This has been confirmed by studies of liver cells of leukemia patients with Australia antigen (18). Patients who are transfused with blood containing Australia antigen will often develop hepatitis accompanied by Australia antigen in the blood (19). Isolated and partially purified Australia antigen has been transmitted to nonhuman primates (infant African green monkeys) and passaged two times. The amount of antigen present in the final monkey is greater than would have been expected by simple dilution (20).

Liver cells from patients with Australia antigen which contain material which reacts with fluorescent anti-Au(1) have been grown in tissue culture. After six passages of the cells the tissue-culture cells or supernatant were still found to contain Australia antigen in their nuclei and the tissue-culture fluid contained Australia antigen (detected by radioimmunoassay) (21). There have been many epidemiologic studies in which the distribution of the Australia
antigen in populations, in disease groups, and in institutions is consistent with
the distribution of an infectious agent.¹

It has been reported that Australia antigen isolated from blood contains
about 5% of RNA (22). This is an extremely small amount if the Australia

| Table II |
| Distribution of Australia Antigen in Serum and Infectious Hepatitis |
| Investigator(s) | Year | n | Serum hepatitis | n | Infectious hepatitis |
| London et al. | 1968 | 41 | 14 | 34 | 84 | 11 | 13 |
| Okochi and Murakami | 1968 | 101 | 13 | 13 | 66 | 10 | 15 |
| Vierucci et al. | 1968 | 8 | 3 | 58 | 80 | 0 | 8 |
| Hirschman et al. | 1969 | 24 | 2 | 8 | 112 | 18 | 16 |
| Gocke and Kavey | 1969 | 34 | 28 | 82 | 15 | 7 | 46 |
| Wright et al. | 1969 | 43 | 21 | 49 | 12 | 3 | 25 |
| Nordenfelt and Kjellen | 1969 | 13 | 8 | 61 | 8 | 2 | 25 |
| Soulier et al. | 1967 | 16 | 9 | 56 | 29 | 0 | 0 |
| Cossart and Vahrnian | 1969 | 13 | 7 | 54 | 66 | 31 | 47 |
| Bals et al. | 1970 | 35 | 34 | 97 | 126 | 83 | 78 |
| Sutnick et al. | 1970 | 75 | 48 | 64 | 71 | 22 | 30 |
| Prince et al. | 1970 | 159 | 101 | 63 | 129 | 71 | 58 |

* n = number of sera tested.
‡ Frequencies of Au(I) as determined by immunodiffusion.
§ Prospective study of post-transfusion hepatitis, weekly samples after exposure.

antigen is a complete virus. It is still not certain if this is a portion of the particle or a contaminant, although the presence of the RNA has been confirmed by other methods (23).

If the RNA finding continues to be supported by additional studies, and if it is assumed that RNA is distributed about equally in each of the particles, then it can be estimated that sufficient nucleic acid is present in each particle to code for only one average-size protein. The molecular weight of Au(I)¹ is

about $3 \times 10^6$, and $5\%$ of this is 150,000. If it is assumed that the RNA is single stranded, then there would be about 450 nucleotides which could code for 150 amino acids. If the RNA is double stranded, then it could code for one-half the number of amino acids. An average protein contains about 100 amino acids.

**TABLE III**

<table>
<thead>
<tr>
<th>Location</th>
<th>n*</th>
<th>Australia</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Philadelphia</td>
<td>7</td>
<td>4</td>
<td>57.1</td>
</tr>
<tr>
<td>Rochester, Minn.</td>
<td>31</td>
<td>3</td>
<td>9.7</td>
</tr>
<tr>
<td>Tokyo</td>
<td>191</td>
<td>21</td>
<td>11.0</td>
</tr>
<tr>
<td>Siena, Italy</td>
<td>30</td>
<td>6</td>
<td>20.0</td>
</tr>
<tr>
<td>New Haven</td>
<td>53</td>
<td>17</td>
<td>32.1</td>
</tr>
<tr>
<td>New York</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chile</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sweden</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Australia</td>
<td>53</td>
<td>2</td>
<td>3.8</td>
</tr>
<tr>
<td>Switzerland</td>
<td>5</td>
<td>1</td>
<td>20.0</td>
</tr>
<tr>
<td>Spain</td>
<td>22</td>
<td>11</td>
<td>50.0</td>
</tr>
<tr>
<td>Austria</td>
<td>22</td>
<td>10</td>
<td>45.5</td>
</tr>
<tr>
<td>Oxford, England</td>
<td>38</td>
<td>10</td>
<td>26.3</td>
</tr>
<tr>
<td>London, England</td>
<td>68</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>London, England</td>
<td>1</td>
<td>17*</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>560</td>
<td>103</td>
<td>18.4</td>
</tr>
</tbody>
</table>

* n = number of sera tested.

Sherlock et al. reported on 17 patients with chronic liver disease in London who had Australia antigen, and also stated that none of 68 with "active chronic hepatitis" were positive. The overall frequency in all chronic hepatitis patients was not stated.

Hence the Australia antigen could code for about one regular protein or a few smaller ones, and the remaining material would have to come from the host. This is unusual for a typical virus.

It is also possible that the RNA is not uniformly distributed throughout all
the particles, and that only a few particles have a full complement of RNA. If this were so, then it could be possible to separate the particles containing RNA from the empty ones using gradient density centrifugation. This has not happened in the studies which have been completed.

Fig. 1. Electronmicrograph of Australia antigen. The particles are about 200 Å in diameter.
Irrespective of any theoretical considerations, it is of practical importance to regard Australia antigen as if it were a virus since isolated Australia antigen is highly infectious and persons working with it may contract hepatitis if precautions are not taken.

Hence, the hypothesis that Au(1) is a virus has not been ruled out; in fact, there is much to support it. However, there are some unusual features of this (apparently) infectious agent which we shall discuss in the next section.

Australia Antigen as a Protein Polymorphism.—Our search for Australia antigen began as an investigation of inherited serum protein polymorphisms, and we have continued to test the hypothesis that Australia antigen is such a polymorphism.

Ford (24) has defined polymorphism as “the occurrence together in the same habitat of two or more (inherited) discontinuous forms of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutations.” This implies, for biochemical and immunological traits, that the forms are easily distinguishable from each other, that the genetics determining the presence of the several forms is explicit, and that the frequency of the least common form is greater than could be maintained by recurrent mutation. Examples of polymorphisms are the blood groups (ABO, Rh, etc.), the sickle-cell hemoglobin polymorphism, and a large series of serum proteins (i.e. haptoglobins, transferrins, Gc, Ag [lipoproteins], etc.). The implication is that there are differences in selective values for each of the genotypes in these systems and that this results in the maintenance of the alternate genes in the populations. (For further discussion, see reference 25.)

Irrespective of any theoretical considerations, the existence of serum protein polymorphisms implies that patients who receive transfusions are almost certain to receive protein variants which they themselves have not inherited or acquired. This led us to investigate the serum of transfused patients for the presence of antibodies against polymorphic proteins (26) and in this way the Ag system (inherited antigen differences on the low density lipoproteins) was discovered (27). A continuation of these investigations led to the discovery of the Australia antigen.

The features of Australia antigen which are similar to those of a serum protein polymorphism are summarized here.

(a) Australia antigen has many chemical and physical characteristics of a serum protein. It migrates by electrophoresis in the alpha position on agar gel; it stains with protein and lipid stains. Its density of about 1.21 (28) is greater than that of low density lipoproteins, to which it bears similarities (29), but its density is less than most other serum proteins.

(b) Millman and his colleagues have isolated Australia antigen and purified it by column separation, sucrose and cesium chloride density gradient centrifugations, and digestion with proteolytic enzymes which can hydrolyze any remaining serum proteins (28). The isolated Australia antigen does not have any
detectable serum protein as measured by immunologic techniques: when this isolated material is concentrated 10-fold it does not react with high titer antiserum to human serum components. When purified Au(1) is treated with the detergent Tween 80, it partially dissociates into soluble components (23). These released products appear to be serum proteins including IgG (both heavy and light chain), complement, beta lipoprotein, transferrin, and albumin. Traces of RNA (about 5%) are also found. The nature of the remaining pellet, which probably is some kind of core material, is not known.

Although it could be argued that these proteins are nonspecific contaminants, it appears more likely that the Australia antigen particle itself actually is made up of human serum proteins, i.e., “host” material although not necessarily from the host from which the Australia antigen was extracted.

(c) There are large numbers of apparently normal people (1-20%), living primarily in southeast Asia and the tropics, who have detectable and sometimes large amounts of Australia antigen in their blood. However, the antigen is quite rare in normal U.S. populations (~0.1%). This is similar to the presence of an inherited serum protein variant in high frequency in some populations but not in others (i.e., serum albumin variant “Naskapi” is common in many northern Indian tribes but absent in people of European origin).

(d) In the areas where it has been tested, the segregation of Au(1) in families follows a pattern of simple autosomal recessive inheritance (30-32). This simple form of inheritance is compatible with the definition of a polymorphic system.

(e) The relation of antigen to antibody does not appear to be typical of that expected of a simple infectious agent. The peculiarities of antigen and antibody behavior is most striking in the studies of transfused thalassemia patients we have completed in collaboration with Dr. Vierucci of Florence, Italy. Several of these transfused patients (many of whom have been followed for up to 7 yr) consistently have antigen and others consistently have antibody, i.e., a transfused patient may “inherently” have either antigen or antibody. This is analogous to the Ag lipoprotein polymorphism in which transfused patients may have a specific antigen or a specific antibody but not both.

CONCLUSIONS

There is no reason to assume that these two hypotheses, (a) Australia antigen is an infectious agent, and (b) Australia antigen is a serum protein polymorphism, are mutually exclusive. Since neither has been rejected, they can be combined to make a third hypothesis, namely, that Australia antigen is an infectious agent which causes hepatitis in some people infected with it and that it has the characteristics of an (inherited) serum protein polymorphism. This concept may generate some interesting hypotheses. For example, since this “new” agent is construed to be a polymorphism one can conjecture that the “host” protein on the agent is not necessarily that of the host from which it is
isolated, but could be related to the previous host or hosts, i.e., it could be an isoantigen. If this concept is correct, then we would expect to see interactions between the infectious agent and the host which are analogous to transfusion reactions. In blood transfusion, if the patient is blood group A and receives type A blood, no antibodies will develop. If the patient receives type B, then antibodies will form and a transfusion reaction can occur. Similarly, if a host is infected by an agent which has the same serum protein specificity as the host, no antibody will form and presumably invasion by the organism can occur. If the specificities are different, then antibodies will form and this may or may not result in invasion and in symptoms of one or more different kinds of disease. This could include antigen-antibody complexes.

Finally, we have to consider the likelihood that Australia antigen is not unique and that other diseases are related to organisms which might also have properties of polymorphisms; kuru and scrapie are two diseases which come to mind. Because of the relationship between leukemia and Australia antigen (33), it is useful to consider that the postulated agent for this disease may fall in this “class” of infectious agents. We propose calling agents of this postulated class “Icrons.”

**SUMMARY**

There is considerable data to support the hypothesis that Australia antigen is an infectious agent that causes hepatitis in man.

(a) Association with acute viral hepatitis.
(b) Association with chronic hepatitis.
(c) Virus-like appearance under the electron microscope (200-A particles).
(d) Transmission of Au(1) from man to man.
(e) Transmission and passage of partially purified Au(1) to an animal host (infant African green monkey).
(f) Localization [with fluorescent anti-Au(1)] of Au(1) in the nuclei of liver cells of patients with hepatitis and/or Au(1) in their blood.
(g) Distribution of Au(1) in institutions, disease groups, and populations is consistent with the distribution of an infectious agent.
(h) RNA identified in Au(1) particles isolated from blood.
(i) Apparent replication of Australia antigen in tissue cultures of human liver cells.

There is also considerable evidence that Australia antigen has many of the characteristics of a serum protein polymorphism. Since neither of these hypotheses has been rejected they can be combined to make a third hypothesis, namely, that Australia antigen is an infectious agent which causes hepatitis in some people infected with it and that it has the characteristics of an (inherited) serum protein polymorphism. We propose calling agents of this postulated class “Icrons.”
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Genes, Viruses, and the Immune Response

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ABSTRACT

Blumberg, Baruch S., London, W. Thomas, Sutnick, Alton I.: Genes, viruses, and the immune response. Amer. J. Clin. Path. 56: 265-269, 1971. Many of the findings ascribed to autoimmunity on the basis of experimental studies could be due to isoimmunity. By this is meant an antibody to an antigenic determinant which is present in some members of a species but not in the particular individual in whom the antibody is detected. The Ag system involves inherited antigenic specificities on the low density lipoproteins. Antibodies against these determinants may form in patients who receive many transfusions or in pregnant women. The role of these antibodies in disease is discussed. The Australia antigen is intimately associated with what appears to be an infectious agent which causes hepatitis in some people infected with it. In addition, it has many features of a serum polymorphism, and isoantibodies may form against it in people infected by transfusion or other means. The relation of these two systems to "autoimmune" phenomena is discussed.

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antibody to an antigenic determinant which is present in some members of a species but not in the particular individual in whom the antibody is detected. The erythrocyte isoantibodies (anti-A, anti-B) are examples of these. An additional consideration is that the antigen in question may also be present in members of other species. Attempts have been made to identify such specificities in bacteria and viruses.

The usual practice in testing for autoantibodies is to use as an antigen tissue from another member of the same species, and in some systems, tissue from another species. Tissue from the same individual has been used only rarely. Therefore, the antibodies which are detected could be either isoantibodies or heteroantibodies. Even if a reaction between an antibody and an antigen from the same individual is detected, the primary antigenic stimulus may have been an isoantigen, with the resulting antibody cross reacting with an antigen in the host.

I would like to discuss two isoimmune systems we have studied and point out some features that may pertain to viruses and the immune process.

Serum protein polymorphisms are inherited differences in serum proteins; the gene frequencies are such that two or more of the phenotypes are relatively common in the population under study. Many of these have been described, i.e., serum haptoglobins, transferrins, Gc protein, gamma globulin, etc. We reasoned that some of the polymorphisms that had already been discovered or some unknown polymorphisms could elicit an antigenic response if one human were immunized, as by transfusion, with the blood of another human who had inherited a protein antigenically different from his own; that is, an isoantibody would form in the transfused patient. This hypothesis was tested using the Ouchterlony (double diffusion in agar gel) method in which the sera of transfused patients were tested against a panel of normal sera. A serum which contained a precipitating antibody that reacted with some, but not all, the sera in the panel was found. There appeared to be only a single class of precipitins, and these were found to react with the serum low density lipoproteins. This was termed the "Ag system." It is important to emphasize that this was the only protein against which precipitating antibodies were detected.

We subsequently were able to show that these specificities were inherited as simple autosomal dominant traits and that their frequencies varied enormously from population to population. Since these initial studies, there have been extensive investigations by a series of European investigators, including Hirschfeld, Vierucci, Butler, Morganti, and others, and at least eight specificities of the lipoprotein have been identified. The genetic studies which have been published indicate that these traits are controlled by alleles segregating at three or more closely-linked loci. More family studies are required to substantiate these genetic interrelations, but the present status indicates a system which is genetically similar to the CDE, Rh locus for the erythrocyte antigens.

We can draw several conclusions from these and other findings related to the Ag system.

1. In testing for autoantibodies in humans it is unusual to test the antibody present in the blood against the individual's own tissue (i.e., his own thyroid, muscle, gamma globulin, etc.), but rather the blood is tested against the tissue of some other individual of the same species. If this approach had been used, the presence of the antibodies against the lipoprotein could have been construed as an autoantibody.

2. Not every individual who is transfused develops antibody against the lipoprotein determinants he does not possess, and the factors resulting in antibody formation are now being evaluated. When an individual does develop an antibody, then the specificity of this antibody is in part genetically determined.

(a) An antibody will not, in general,
form against a lipoprotein determinant that the antibody-former has itself inherited.

(b) The antigenic determinants in the donor blood are genetically determined, and the frequency of the genes varies from population to population. Hence, the genetic makeup of the population will determine the nature of the antigenic risk to which the transfused patient is exposed.

(3) Not only transfused patients develop antibodies against lipoproteins; pregnant women immunized against determinants which their unborn children have inherited from their fathers, but not their mothers, may develop detectable antibodies. Ag antigenic determinants have also been found in primates.

(4) Many patients in whom antibody develops are repeatedly transfused with blood which contains lipoprotein determinants which form precipitates in vitro. This would appear to be the classic situation for the development of antigen–antibody disease of the kind which has been postulated to be related to the pathogenesis of autoimmune disease. Several conclusions have emerged from studies of patients transfused in this manner. Continued transfusion of patients who have developed antibody does not result in a cataclysmic event although there may be some evidence of inflammatory reaction (i.e., fever). Despite this, there could be long term effects of continued exposure. Thalassemia patients who develop antibody and are subsequently transfused have a significantly greater mortality than thalassemia patients who do not develop an antibody. These findings, although statistically significant, are based on a small sample, and there may be some trivial explanation for the finding. However, taken at its face value they do not rule out a pathologic effect of this antigen–antibody reaction.

(5) Lipoproteins are found in and on many tissues and cells; for example, the transplantation antigens contain lipoproteins. Very little has been done on this aspect of lipoproteins, but this would appear to be a fruitful area for future research on disease relations.

I would now like to discuss the Australia antigen (Au) system, which was discovered in the same way as the Ag (lipoprotein) system. It introduces a third element for consideration, namely, an infectious agent, in addition to the two already considered, that is, antigen–antibody interactions and genes. There has been more interest in the Australia antigen system than in the Ag (lipoprotein) system because Au is associated with hepatitis and that the Australia antigen test can be used for the diagnosis of hepatitis and the detection of hepatitis carriers. However, there are additional features which pertain to the isoantibody question. Australia antigen was discovered as a consequence of the continuation of the study which originally led to the finding of the Ag (lipoprotein) system. After the discovery of the original Ag serum, the search for additional antisera was continued. This resulted in the detection of additional anti-lipoprotein antisera, including the new Ag specificities. In 1963, a precipitin which was clearly different from those previously found was discovered in the serum of a hemophilia patient. The initial reaction was with only a single member of the panel against which it was tested and, since the blood was from an Australian aborigine, it was termed “Australia antigen.” In one of our initial studies, we showed that the Australia antigen was distinct from the Ag lipoprotein system, but despite this it was also similar in some respects. In addition to studies on the association of Australia antigen with disease, we continued to investigate the characteristics of the system as a polymorphism. The results have been published and reviewed elsewhere, and will be summarized briefly here.

Australia antigen has many characteristics of an infectious agent which can cause clinical hepatitis (both acute and chronic) in many patients infected with it. It is associated with acute and chronic hepatitis in many populations, it has the electron
microscopic appearance of a virus (200 A diameter), and it has some physical characteristics of a virus.\(^3,21\) It has been transmitted from man to man by transfusion, and the recipients may develop hepatitis.\(^23\) Isolated and partially purified Australia antigen has been transmitted and passed to African green monkeys.\(^28\) In the human and animal transmission studies, the amount of material increased enormously in the infected individuals, i.e., it acts as if it were both infectious and a reproducing agent. Using fluorescent anti-Au\(_4\), material with the same antigenic characteristics as Australia antigen has been identified in the nuclei of patients with hepatitis and Australia antigen in their peripheral blood.\(^10,22\)

The distribution of Australia antigen in patients, populations, and institutions is compatible with the distribution of an infectious agent.

There are two findings which do not support the virus hypothesis. Nucleic acid has not been detected in the preparations isolated from the peripheral blood, and Australia antigen has not been reported to grow on tissue culture.\(^21\) The failure to find nucleic acid does not rule out the possibility that Australia antigen is a virus. For example, the nucleic acid could be present in only a small percentage of the particles and the material would still be infective. These findings and other unusual characteristics of this agent raise the question that "Australia antigen" may in fact be another kind of an infectious agent whose characteristics are similar to but also different from those of viruses.

Antibodies to Australia antigen may develop in humans (and animals) to whom it is transmitted by transfusion or by other means. Some of the humans will develop hepatitis and others will not. Both antibody and antigen may be found in the same individual at different times. At least seven different kinds of Australia antigen antisera have been identified. These may be defined by three or more specificities. An individual may at some time have in his blood an antigen of one determinant and an antibody against another determinant at the same time.\(^24\) Complexes of antigen and antibody may be found in some individuals and patients. The distribution of these complexes has not been studied systematically. Australia antigen also appears to occur commonly in some patients with forms of periarteritis.\(^10\)

There are striking differences in the responses of different individuals "infected" with Australia antigen.\(^12\) Some develop acute viral hepatitis, others chronic active or persistent hepatitis, and another group may be carriers with no apparent illness. The latter are particularly common in some parts of the tropics. There is an additional group of individuals with certain underlying chronic diseases who, when infected, develop a persistent infection which may be accompanied by chronic anicteric hepatitis. These include Down's syndrome, lymphocytic leukemia, lepromatous leprosy, and others. There is accumulating evidence that these diseases are characterized by an impairment of the immune mechanism related to delayed tissue hypersensitivity as well as antibody formation (see, for example, reference 1).

In the populations in which Australia antigen is common it is possible to view the distribution as analogous to a polymorphism. Three studies \(^6,13,18\) support the hypothesis that susceptibility to persistent infection with Australia antigen is inherited as a simple, autosomal recessive trait. The individuals homozygous for this postulated Au\(_{-}\) gene appear to be asymptomatic carriers of the hepatitis agent.

Hence, these two systems have features which may be considered relative to what have been called autoimmune diseases. In the two systems the isoantibodies may be induced by transfusion, by a fetus, or (in

\(^*\)Since the presentation of this paper, RNA has been identified in isolated Australia antigen by Nowoslawski and his colleagues, and a form of growth in tissue culture has been shown by Coyne and her colleagues at the Institute for Cancer Research.
the case of Australia antigen) by infection.
The individual’s genetic makeup has a bearing, both direct and indirect, on his ability to produce antibody and on the specificity of the antibody produced. The antigen, antibody, and antigen–antibody complexes may or may not be associated with disease.

In the Ag system the presence of the antibody appears to be determinable, but the characteristics of the disease process are unknown. In the Australia antigen system the agent associated with the antigen may cause hepatitis, but not everyone infected with it manifests the disease. There has been considerable interest in the role of Australia antigen–antibody complexes in the pathogenesis of diseases other than hepatitis.11

The conventional views on autoimmune disease do not encompass the kinds of data I have discussed, or many of the extensive findings of Mellors and others we will hear from today. It is now important to make new hypotheses for testing, and perhaps this symposium will generate some of these.

References
Viral hepatitis, Au antigen, and hope for a vaccine BARCH US B. BLUMBERG, M.D., Ph.D.

Our discovery of the Australia antigen has raised hopes that an effective hepatitis vaccine may be feasible. The studies we completed during 1967 and 1968 suggested that the antigen is intimately associated with the infectious agent that causes "viral" hepatitis. These early observations also pointed up striking differences in the way individuals respond to infection with what seem to be the same (or very similar) agents. Responses seem to fall into the following four categories:

- Acute hepatitis with transient appearance of the antigen;
- Chronic hepatitis with persistent antigen;
- Antibody either after acute disease or without any evident disease;
- A persistent and largely asymptomatic carrier state. Carriers are particularly common in certain tropical areas and in Southeast Asia, and susceptibility to this state is probably an inherited trait. All these findings had several important effects on our ongoing efforts to achieve a vaccine.

Developing the Vaccine

The antigen we found in asymptomatic carriers appeared to be identical to or very similar to that found in hepatitis patients. Further, some of the apparently asymptomatic carriers had enormous amounts of antigen; we estimated that as much as 1% of some carriers' serum protein was made up of Australia antigen. Thus the antigen from asymptomatic carriers might prove to be a source of vaccine material that would require no tissue culture for preparation.

Drs. Irving Millman and Blumberg developed a method for isolating Australia antigen from serum. We used column separations, density gradient centrifugation, and proteolytic digestion to remove all the other serum constituents. We also devised methods for inactivating infectivity. We soon learned that material isolated in this manner produced antibody in rabbits, guinea pigs, mice, goats, and nonhuman primates. We also learned indirectly that it could produce antibodies in humans; we found that many individuals who had inadvertently received Australia antigen in transfused blood developed high titers of antibody after immunization. At this point, we didn't feel we knew enough about the Australia antigen to consider extensive clinical trials. But useful information from other observations is accumulating.

Dr. Saul Krugman and his colleagues have reported some controversial studies in which they used boiled serum containing Australia antigen as a vaccine. The results of their preliminary work are rather difficult to interpret. First, their subjects were mentally retarded children, including some with Down's syndrome, who often have an unusual immune mechanism with respect to Australia antigen. And in their initial experiments, Dr. Krugman and his associates did not study a control group.

Nevertheless, Dr. Krugman's results appear to support the view that a vaccine could be protective. However, as Dr. Krugman has pointed out, these results are preliminary. It is unlikely that extensive clinical trials will be conducted before more information is obtained on the nature of the Australia antigen and on the potential hazards of the vaccine.

Our next step is to examine some of the immunologic features of Australia antigen and the host reaction to it. Both of these will have a bearing on the development and use of the vaccine. All the information so far indicates that antibodies could protect against hepatitis.

In our first papers on Australia antigen we pointed out that the antigen has multiple specificities. Subsequently we used the terms "anti-Au (1)" and "anti-Au(2)" to designate specific antisera, referring to the antigenic determinants as a, b, c, etc. Subsequent systematic studies by Dr. G. L. Le Bouvier resulted in the identification of additional specificities in human antisera; several of these—anti-x and anti-y, for example—have been extensively studied. Dr. V.K. Raunio and his colleagues have also shown that some individuals can have persistent antigen in their blood and at the same time have antibody against specificities different from those of the antigen.

Any design for a vaccine would of course have to allow for multiple specificities, and specific vaccines may be required during some hepatitis outbreaks. Since nearly all the specificities identified so far have at least one specificity in common, it may be possible that one or perhaps only a small number of vaccines will provide good control for most outbreaks in
any given geographic region.

In his pioneer studies on the effects of transfusing blood containing Australia antigen, Dr. Kazuo Okochi found that individuals who developed antibody have a significantly lower incidence of hepatitis. These studies gave no information on the course of the disease in patients who contracted it despite their antibody. In a recent summary of his extensive experience with the transfusion of blood containing Australia antigen, Dr. David J. Gocke also noted that none of the patients who initially developed antibody contracted clinical hepatitis—another indication of the protective power of antibodies against the disease.

During the past year, high-titer human antibody against Australia antigen has become available. Gamma globulin extracted from these antisera have been used to test the efficacy of passive antibody transfer. Individuals inadvertently stuck with needles contaminated by Australia antigen have been given the hyperimmune gamma globulin prophylactically. To my knowledge, reports of controlled studies have not yet been published, and cannot be fully evaluated until they are, but the preliminary information so far available is encouraging; it seems to indicate that the hyperimmune gamma globulin is protective.

In another recently published study, Dr. M.E. Conrad and his associates report on an extensive clinical trial of the effectiveness of gamma globulin in preventing hepatitis. Their subjects were U.S. soldiers serving in Korea, and the gamma globulin prevented a significant amount of viral hepatitis associated with Australia antigen as well as that in which Australia antigen was not identified. The batches of gamma globulin used in the Korean study contained detectable amounts of anti-Au(I), whereas many batches of commercial gamma globulin have no detectable antibody. So it is possible that the Korean program was successful because the gamma globulin contained the antibody.

Methods for production of the vaccine have been developed, and there is indirect evidence that vaccine could be protective. What now remains to be done is to increase our understanding of the nature of Australia antigen in order that appropriate animal and, subsequently, clinical trials can be devised.

Australia antigen has characteristics that are not completely typical for a virus. In particular, at least according to our studies, it appears to contain considerable amounts of host material and, if our genetic studies are correct, there may be reactions between the infectious agent and potential hosts similar to transfusion reactions.

Further, as already noted, the response to infection varies enormously from individual to individual; this, at least in part, is related to the specific character of the immune mechanism of the patients infected. Hence, some individuals may be at risk and will benefit from vaccination, whereas others do not require vaccination but can muster their own defenses. Much of our work is now directed to the discovery of methods for identifying these different individuals.

Detecting the Carriers

On the basis of retrospective studies it has been said that about 25% of cases of post-transfusion hepatitis can be eliminated by the use of the Australia antigen test. In Dr. Gocke’s extensive series he found that the more serious cases of hepatitis appear to be those associated with the transmission of positive units of the blood. For example, of 84 patients who were recipients of Au-positive blood and were followed, 28 developed hepatitis with icterus. Of 94 receiving Au-negative blood, only two developed icteric

continued
hepatitis. If Dr. Gocke's studies are borne out in other investigations it would indicate that, at least in some patient populations, the majority of the serious cases of post-transfusion hepatitis could be eliminated.

Routine testing of donor blood for Australia antigen has been carried on in Philadelphia since 1968, probably longer than in any other community. By a fortunate circumstance, Dr. John R. Senior and his colleagues at the Philadelphia General Hospital had, in the 1960s, conducted an intensive follow-up of patients receiving transfusions to determine the frequency of post-transfusion hepatitis at that hospital. They found that post-transfusion hepatitis (both icteric and nonicteric) occurred in about 18% of the transfused patients.

After the Australia antigen testing had been in progress at PGH for about one year (using the immunodiffusion method, which is less sensitive than other methods now available), a similar follow-up study was conducted using the same criterion for case finding as in the pre-testing study. They found that the amount of post-transfusion hepatitis had been decreased to about one third of that found prior to the use of the test. During this period the source of the donor blood had not been changed. If these findings are confirmed in similar studies in other institutions, then it would indicate that more than half of post-transfusion hepatitis could be eliminated.

Although it is very difficult to get reliable information on how much post-transfusion hepatitis there is in the U.S., it seems reasonable to estimate a savings of about 1,400 lives and $200 million to $300 million a year if the Australia antigen test were used universally. The American Red Cross, and all blood banks approved by the American Association of Blood Banks, now require the testing of blood for Australia antigen. Therefore it should be possible to evaluate the usefulness of this test within the next few years.

Hepatitis in the Mentally Retarded
Many of our early observations on Australia antigen and hepatitis were made on patients with Down's syndrome. The information gained may be of importance in the care of these patients, since it appears possible to prevent or control the epidemics of hepatitis that occasionally spread through institutions for the mentally retarded.

Australia antigen is more prevalent among patients with Down's syndrome living in large institutions than it is among other mentally retarded patients in the same institutions. Since this observation was made in 1966, it has been confirmed in many laboratories. In Down's syndrome patients, Australia antigen is associated with chronic anicteric hepatitis, as evidenced by small but significant abnormalities in SGPT, SGOT, cephalin flocculation, thymol turbidity, and BSP retention; in addition, there are abnormalities on liver biopsy.

Although the antigen is common in Down's syndrome patients residing in large institutions, it is much less common among those in small institutions. We have not found the antigen in patients living at home because susceptible individuals in large institutions are more likely to become persistently (or repeatedly) infected.

The preventability of the disease is implicit in these data. For one thing, it is possible that if reinfections, rather than a single persistent infection, cause chronicity, then removing patients from their contaminated environment could be curative. With the antigen test we can now evaluate how best to prevent or treat hepatitis in these patients. The test also can be used to monitor, and possibly prevent, the epidemics of acute icteric hepatitis that often sweep through large institutions for the mentally retarded, affecting both patients and staff. Here the Down's syndrome patients represent an enormous reservoir of infection.

Dr. Blumberg is associate director for clinical research at the Institute for Cancer Research, Philadelphia, and professor of medicine and medical genetics at the University of Pennsylvania.

SUMMARY OF EARLY STUDIES ON THE FREQUENCY OF AU ANTIGEN IN ACUTE VIRAL HEPATITIS

<table>
<thead>
<tr>
<th>Investigators/Date</th>
<th><em>Serum</em></th>
<th>Hepatitis %</th>
<th>No. Pts. Studied</th>
<th>Infectious *</th>
<th>Hepatitis %</th>
<th>No. Pts. Studied</th>
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<td></td>
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<td></td>
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<td>Hirschman et al, 1969</td>
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<td>Bals et al, 1970</td>
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<td></td>
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<td>Prince et al, 1970</td>
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Reduction of post-transfusion hepatitis by exclusion of Australia antigen from donor blood in an urban public hospital

JOHN R. SENIOR
ALTON I. SUTNICK
EUGENE GOESER
W. THOMAS LONDON
MIRIAM B. DAHLKE
BARUCH S. BLUMBERG

Abstract: Post-transfusion hepatitis occurred in ten of 56 (17.9 per cent) patients in 1963-1964, and in 14 of 78 (17.9 per cent) patients in 1968-1969 at Philadelphia General Hospital (PGH). Since November 1969 all donor blood has been tested for Australia antigen (Au) by immunodiffusion and later by counterelectrophoresis, and no positive units have been transfused. All patients were screened by stringent criteria to exclude any pre-existing liver disease and had repeated follow-up examinations after transfusion, including clinical examination, as well as serial serum glutamic pyruvic transaminase (SGPT) and Au determinations. Among 204 patients receiving Au-negative donor blood who could be followed adequately for six months, 12 (5.9 per cent) hepatitis patients (four icteric) were detected. These results indicate a two thirds reduction from the 18 per cent incidence in post-transfusion hepatitis at PGH found on two previous studies. Au testing to exclude positive donors and administering only Au-negative blood, and the changes in composition of the donor population which resulted, were effective in reducing the incidence of post-transfusion hepatitis.

KEY INDEXING TERMS
Australia antigen
Hepatitis
Transfusion
Liver disease
Post-transfusion hepatitis

It has been recognized that hepatitis following blood transfusion occurs more frequently\(^1,2\) than earlier reports had indicated.\(^3,4\) The source of blood probably is the most important determinant of the hepatitis risk after transfusion.\(^5-7\) Since the discovery of Australia antigen (Au, HAA, HBHg),\(^8,9\) it has been possible to identify high or low risk sources.\(^10\) Blood from a group with a high percentage of Au-positive donors carries a high risk of transmitting hepatitis.\(^11\)

In a study performed in 1963-1964 at the Philadelphia General Hospital (PGH),\(^1\) we observed prospectively patients on the obstetrical-gynecological service for occurrence of hepatitis after blood transfusion. A hepatitis frequency...
REDUCTION OF POST-TRANSFUSION HEPATITIS

of 17.9 per cent was found. In an effort to confirm and extend these earlier observations, recipients of whole blood, plasma, packed red blood cells, and nontransfused control patients were followed at the Hospital of the University of Pennsylvania (HUP) and PGH. These two hospitals serve distinctly different population segments. The PGH patients come almost exclusively from the poor, inner city population, while the patients at HUP are referred from a wide area around Philadelphia. Post-transfusion hepatitis in patients on medical, surgical, and gynecologic services at PGH during the repeat study in 1968-1969 confirmed the almost 18 per cent incidence of hepatitis (Table 1).

We report our experience with a program of testing donor blood units at these hospitals, particularly the effect of withholding all positive units in producing a significant reduction in the incidence of post-transfusion hepatitis at PGH.

Materials and Methods

Beginning in September 1968 and continuing until April 1971, patients were accepted for study from all services in both hospitals, male and female, of any age, and with a variety of disease entities (Table I). We followed prospectively after transfusion 551 patients who were willing to be followed, who had no history of prior transfusion, hepatitis contact, heavy alcohol intake, drug addiction, exposure to hepatotoxic agents or drugs, and no history or clinical indication by physical examination of past or present liver disease, malignancy or sickle cell disease. Control patients from neighboring beds on the same services, of similar age, sex, and disease, but not transfused, were followed concurrently in the same manner, as was done previously. Because of the suggestion that packed red blood cells might be safer than whole blood, other groups of recipients of only packed cells were also followed.

Determinations of serum glutamic pyruvic transaminase (SGPT), serum glutamic oxalacetic transaminase (SGOT), bilirubin, alkaline phosphatase, protein electrophoresis, and testing for Au and antibodies to Au (AuAb) were performed prior to or at the time of transfusion, and only patients with normal values were followed. All tests on patients from both hospitals were performed at the Pepper Clinical Chemistry Laboratory of HUP. Follow-up of patients was done at intervals of 1, 2, 3, 4, 6, 8, 10, 13, 17, 21, and 26 weeks after transfusion; at these times history, physical examination, and laboratory determination of serum glutamic pyruvic transaminase (SGPT) Au and AuAb were performed.

The criterion for the diagnosis of anicteric hepatitis was an elevation of SGPT above 100 International Units (I.U.), otherwise unex-

| TABLE I |
| HEPATITIS AFTER TRANSFUSION OF BLOOD PRODUCTS |

<table>
<thead>
<tr>
<th>Study</th>
<th>Product</th>
<th>Recipients</th>
<th>Hepatitis (Icteric)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>At PGH:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1963-1964</td>
<td>Whole blood, plasma</td>
<td>56</td>
<td>10 (0)</td>
<td>17.9</td>
</tr>
<tr>
<td>1968-1969</td>
<td>Whole blood, plasma</td>
<td>78</td>
<td>14 (4)</td>
<td>17.9</td>
</tr>
<tr>
<td>1968-1969</td>
<td>Packed red cells only</td>
<td>29</td>
<td>3 (0)</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>After Au Screening-Exclusion Program</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1968-1969</td>
<td>Whole blood, plasma</td>
<td>204</td>
<td>12 (4)</td>
<td>5.9</td>
</tr>
<tr>
<td>Nontransfused Control Patients Followed Similarly</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1963-1964</td>
<td></td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1968-1970</td>
<td></td>
<td>62</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>At HUP:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1968-1969</td>
<td>Whole blood, plasma</td>
<td>64</td>
<td>3 (1)</td>
<td>4.7</td>
</tr>
<tr>
<td>1968-1970</td>
<td>Frozen, washed red cells</td>
<td>33</td>
<td>0</td>
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</tr>
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</table>
plained, occurring on at least subsequent occasions in a patient with previously normal values and persisting for at least three weeks. Hospitalization for further studies and liver biopsy was recommended for these patients. All patients with this degree of serum enzyme activity also showed findings of hepatitis when liver biopsy could be performed, and transient elevations in bromsulfonphthalein (BSP) retention, as previously reported.\(^1\)

Donor blood from HUP and PGH was tested for Australia antigen at The Institute for Cancer Research, Fox Chase, by the immunodiffusion method.\(^1^3\) Results were communicated to the blood banks by telephone, and positive units, if not yet used, were immediately removed from the blood bank inventory.

Based on early observations that about half the recipients of donor blood containing Au developed hepatitis,\(^1^4,1^5\) we decided in July 1969 that no donor blood found to contain Au should be administered, and that mechanisms would be set up to examine all donor units. By the fall of 1969 complete testing of all donor blood at PGH was being performed and units were released for transfusion only if negative for Au. Thus PGH probably became the first hospital in the United States to implement a full Au screening and exclusion policy. As more test sera became available in 1970, the blood bank at PGH was able to set up its own testing, with both the immunodiffusion and the couterelectrophoresis\(^1^6\) methods; duplicate specimens were sent to Fox Chase for processing as controls.

**Results**

In 1969, before the policy of testing and exclusion was established, a total of 57 patients had received at least one unit of blood containing Australia antigen. Of these patients (Table II), 13 at HUP and seven at PGH died of underlying disease within a few weeks after transfusion; four at HUP and six at PGH did not wish to be re-examined; 14 at HUP and 13 at PGH could be followed satisfactorily for a six-month period. Twelve of the 27 patients (44 per cent) followed for six months showed evidence of hepatitis (three icteric). Seven patients in whom hepatitis developed showed transient detectable Au; one developed antibodies to Au, and four patients had hepatitis without demonstrating either Au or antibodies. Of the 15 patients who did not have evidence of hepatitis, seven developed antibodies to Au, while eight patients had no abnormalities.

<table>
<thead>
<tr>
<th>Subject</th>
<th>No.</th>
<th>Au</th>
<th>AuAb*</th>
<th>Neither</th>
</tr>
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<tbody>
<tr>
<td>Died of other disease</td>
<td>20</td>
<td>7</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Refused follow-up</td>
<td>10</td>
<td>0</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Followed (observed for full six months)</td>
<td>27</td>
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</table>

*AuAb, antibodies to Au, as detected by immunodiffusion.

Eight of the Au-positive donors implicated in possibly transmitting hepatitis were available for further evaluation. All eight showed transaminase abnormalities in the range of SGPT 70-510 I.U. and SGOT 75-240 I.U., as well as elevation in the immunoglobulins (mainly in immunoglobulin G) varying from 2000 to 3800 mg per cent. All had been clinically asymptomatic and denied exposure to hepatitis or drug use when evaluated prior to blood donation.

Early in the study we had noticed a marked difference in the overall frequency of post-transfusion hepatitis between the two hospitals. In 1968-1969, there were 14 patients with hepatitis, of whom four were icteric, at PGH among 78 patients (17.9 per cent) followed for a full six-month period. Hepatitis did not occur in 55 nontransfused control patients. During the same time period, only three cases of hepatitis after transfusion, one icteric, were seen among 64 patients (4.7 per cent) followed at HUP. The correlation of post-transfusion hepatitis with donor antigen frequency became evident when we obtained more results of Au testing of the various donor groups. During 1969-1970, more than 26,000 donor units were tested at both hospitals. Testing was incomplete.
TABLE III
AU FREQUENCY IN DONOR BLOOD UNITS, AT HUP AND PGH

<table>
<thead>
<tr>
<th></th>
<th>1969</th>
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<tbody>
<tr>
<td></td>
<td>Tested</td>
<td>Au</td>
</tr>
<tr>
<td><strong>HUP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor room, club</td>
<td>1735</td>
<td>6</td>
</tr>
<tr>
<td>American Red Cross</td>
<td>2322</td>
<td>4</td>
</tr>
<tr>
<td>AABB*</td>
<td>1502</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>5559</td>
<td>12</td>
</tr>
<tr>
<td><strong>PGH</strong></td>
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<tr>
<td>Donor room</td>
<td>634</td>
<td>6</td>
</tr>
<tr>
<td>Prisoners</td>
<td>1307</td>
<td>41</td>
</tr>
<tr>
<td>American Red Cross</td>
<td>768</td>
<td>0</td>
</tr>
<tr>
<td>Commercial, professional</td>
<td>133</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>2842</td>
<td>49</td>
</tr>
</tbody>
</table>

*Units obtained from other hospitals in the region through the registry of the American Association of Blood Banks.

TABLE IV
AU FREQUENCY IN DONOR BLOOD AT PGH

<table>
<thead>
<tr>
<th></th>
<th>1969</th>
<th>1970</th>
<th>1971</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested</td>
<td>Au</td>
<td>%</td>
</tr>
<tr>
<td>Volunteer donors</td>
<td>0.95%</td>
<td>1.14%</td>
<td>1.31%</td>
</tr>
<tr>
<td>Prison donors</td>
<td>3.14%</td>
<td>1.48%</td>
<td>1.48%</td>
</tr>
<tr>
<td>American Red Cross</td>
<td>0</td>
<td>0.50%</td>
<td>0.06%</td>
</tr>
<tr>
<td>Commercial donors (including professional)</td>
<td>1.5%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1.72%</td>
<td>0.86%</td>
<td>0.54%</td>
</tr>
</tbody>
</table>

Units used (as whole blood, packed cells, fresh frozen plasma, etc.)

<table>
<thead>
<tr>
<th></th>
<th>1969</th>
<th>1970</th>
<th>1971</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Tested/Collected.
†Collected and tested.

in 1969; only 38 per cent of all donor units collected for use at HUP and 58 per cent of all units for PGH were screened, while in 1970 all units were tested before transfusion (Table III). The overall frequency of Au was markedly higher in the PGH donor populations than in HUP populations. By far the highest frequency was observed in the PGH paid prisoner donors (1969, 3.14 per cent; 1970, 1.48 per cent). Volunteer donations by friends and relatives of PGH patients showed a much higher frequency (1969, 0.95 per cent; 1970, 1.14 per cent) than the comparable group at HUP (1969, 0.35 per cent; 1970, 0.21 per cent).

During 1970 and 1971, after donor screening and exclusion of Au-positive units had been fully instituted, only antigen-negative blood was transfused at PGH. It was already evident that screening and excluding Au-positive donors resulted in a marked change in the prevalence of Au in the PGH donor population, prisoner donors (Tables III and IV). We have been able
to follow a total of 204 patients who received only tested Au-negative blood with serial SGPT and Australia antigen determinations for six months after transfusion. Twelve cases of hepatitis (5.9 per cent) occurred in this group, four of which were icteric (Table I), a significant decrease from the previous level (p = 0.000035). The use of packed erythrocytes resulted in only a slightly lower prevalence of hepatitis, and the difference was not significant (10.3 per cent, p = 0.145).

When liver biopsy was performed, all patients with three SGPT elevations to 100 I.U. or more showed findings consistent with mild viral hepatitis and BSP retention greater than 10 per cent at 45 minutes, as had been observed in the previous study.7 Most anicteric patients were either completely asymptomatic or had only vague complaints of anorexia and fatigue. Of the four icteric patients only one required hospitalization. This patient had received 15 units of whole blood; his transaminase rose to 1528 I.U. of SGPT and 1908 I.U. of SGOT, and his serum total bilirubin reached a high of 22.6 mg/100 ml. None of the other three icteric patients showed a total bilirubin of more than 4.0 mg/100 ml; their clinical courses were mild. Maximum transaminase (SGPT) elevation ranged from 235 to 1530 I.U., the average being 607 I.U. for the entire group. Liver histopathology was available from six anicteric patients, and the findings were interpreted as viral hepatitis in each patient by the PGH pathologists, who were without knowledge of the study. Inflammatory cell infiltrates of mild to moderate degree in the periportal and intra lobular areas, mild to moderate degenerative liver cell changes manifested as granularity, ballooning and occasional focal necrosis as well as presence of hyaline (Councilman) bodies were the consistent findings in the tissue specimens. The clinical picture, the laboratory findings, and the biopsy results showed no significantly different features as compared to the findings of previously studied patients who had presented with icteric and anicteric hepatitis after transfusion of blood containing Australia antigen. None showed serum Australia antigen during the follow-up period. In a nontransfused control group of 62 patients from neighboring beds who were selected by the same criteria and followed concurrently the same way, no case of hepatitis was found.

Discussion

In two independently conducted similar studies, separated by a five-year period, we found the frequency of icteric and anicteric hepatitis after blood transfusion to be about 18 per cent among patients at Philadelphia General Hospital.1 A comparable number of patients selected in the same manner and followed during the same time period at the adjacent Hospital of the University of Pennsylvania showed a frequency of less than 5 per cent. The major effort of 1970-1971 was directed toward determining to what extent post-transfusion hepatitis could be reduced at PGH by an Au screening and exclusion program. The 5.9 per cent overall incidence of hepatitis after blood transfusion observed at PGH during 1970-1971 was a marked reduction from the 18 per cent observed in two previous groups of patients. It was comparable to the less than 5 per cent incidence previously observed at HUP in a group of patients who had received untested blood, mainly from donors with a much lower frequency of Au.

As had been shown empirically before Au testing was available, there are certain population groups in which the hepatitis-inducing agent or agents are present more frequently.2,6,17 Early in 1969 we began to collect data about the sources of blood used for transfusion at both hospitals. Blood from paid donors from three Philadelphia prisons, a source upon which PGH is dependent to obtain sufficient blood for emergency needs, had the highest frequency of positive units. Although known addicts were excluded as donors, it is well known that drug use and addiction are high among prisoners and, since they donate for payment, it is likely that many deny drug use, previous hepatitis, or exposure to hepatitis. The cramped, close living conditions in the penal institutions would also facilitate the nonparenteral spread of hepatitis.
REDUCTION OF POST-TRANSFUSION HEPATITIS

from asymptomatic carriers. Thus, prisons appear to constitute a significant hepatitis virus pool in this community.

A marked difference was present in volunteer-donated units. Friends and family members donating for PGH patients had a fivefold higher frequency of Au units than the same kind of donors at HUP. The volunteer group at PGH had a different socioeconomic background from the HUP donor population; PGH patients were almost exclusively from the poor, predominantly black, inner city population. The substandard housing and hygiene, cramped living conditions, and poorer overall health care in this group are other factors that could facilitate the spread of infection. Blood obtained from the American Red Cross showed approximately equal frequencies of positive units at both hospitals.

Our studies and those of others have shown that administration of blood containing Au produces observable hepatitis in almost 50 per cent of recipients. Another 20 to 30 per cent produce antibodies to Au, an indication of some active immunologic response to the agent transfused. The evidence indicates that these antibodies may protect these patients against the disease. A significantly (p <0.05) lower incidence of hepatitis was observed in those developing antibodies after blood containing Au was transfused (Table II). The patients at PGH who developed hepatitis in the combined 1968-1969 and 1970-1971 studies each received an average of 4.3 units of blood, while, in the entire group followed, the average patient received just above 2.5 units. It appears that most of the Au-negative donor blood causing hepatitis originated from the same donor population that had been shown to cause hepatitis in nearly 50 per cent of recipients of Au-containing blood. Sixty-five per cent of the blood transfused to the hepatitis group had originated from prison donors and volunteer PGH donations.

In a recent study of 126 patients at the National Institutes of Health,18 by excluding both commercial donors and all units from Au-positive donors, post-transfusion hepatitis has been reduced to only 3.7 cases per 1000 units transfused. Efforts to reduce further the incidence of transfusion-related hepatitis should proceed in the direction of excluding Au-positive blood and interdicting transfusion of blood from demonstrated high-risk donor groups.

References


HEPATITIS B VIRUS

Subtypes
Additional Specificities of Australia Antigen and the Possible Identification of Hepatitis Carriers

In 1964 (ref. 1), we identified an antibody which is common in the blood of transfused haemophiliacs and which reacts with an antigen (first identified in an Australian aborigine, thus “Australia antigen”) found in some human sera. Australia antigen, Au(1), is found transiently in the sera of many patients with acute viral hepatitis (13 per cent in infectious hepatitis, 34 per cent of post transfusion hepatitis). This association with hepatitis has been confirmed by other workers using reference antisera from our laboratory (refs. 10 and 11, and unpublished results of Vierucci). Okochi and Murakami used an anti-Australia antiserum from a transfused patient which seems to be identical with reference sera we have exchanged. The “SH antigen” recently described by Prince is identical with Australia antigen. On the basis of previous experience with iso-antigens from transfused patients (the Ag system, inherited iso-antigenic specificities on the beta-lipoproteins) we had reason to believe that other specificities could be found or produced by appropriate rabbit immunizations. A rabbit was therefore immunized with the serum of a patient with leukaemic reticuloendotheliosis (C.F.) using the same procedure as in our earlier immunization. The serum from this patient had given an unusual and inconsistent reaction with anti-Au(1) serum. The immunization produced an antiserum (anti-Au(2)) which had at least one specificity in common with anti-Au(1) but one in addition to this. Furthermore, anti-Au(1) was found to have two specificities, only one of which was common to that in the “new” anti-Au(2) rabbit antiserum. Antiserum which gave the same reactions as anti-Au(2) were produced in two other rabbits. These identities and dissimilarities of specificities were demonstrated by the formation of spurs in Ouchterlony immunodiffusion experiments (Fig. 1). Hence the anti-Au(1) identifies at least two specificities of Australia antigen, a and c; and anti-Au(2) identifies at least two specificities, a and b (Table 1).

Ant-Au(1) has been useful in detecting hepatitis carriers and patients with chronic anicteric hepatitis in several situations—for example, Down’s patients with chronic anicteric hepatitis (30 per cent of institutionalized patients), and patients receiving frequent haemodialysis.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Specificities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Au(1)</td>
<td>a, c</td>
</tr>
<tr>
<td>Anti-Au(2)</td>
<td>a, b</td>
</tr>
</tbody>
</table>
for chronic renal disease\textsuperscript{14} (eight of nine patients). It has been less useful in detecting hepatitis carriers among blood donors in the USA where Au(1) specificity is rare (about 0.1 per cent). Au(1) is, however, more common in Japan (about 1 per cent) and much more common in south-east Asia and elsewhere in the tropics (3-20 per cent). Okochi and Murakami\textsuperscript{16} have used anti-Au(1) antiserum to detect carriers among blood donors and have shown that patients transfused with Au(1) may develop hepatitis associated with Australia antigen, or antibodies against Au(1) with no clinical disease.

The distribution of reactions with anti-Au(2) is very interesting in this regard (Table 2). The frequency of reactors is very high in acute viral hepatitis, in institutionalized patients with Down’s syndrome (in whom chronic anicteric hepatitis is very common), in employees of institutions for mentally retarded patients who are known to have a high frequency of hepatitis, and in thalassemia patients who have received very large numbers of transfusions. The frequency in apparently normal blood donors approximates that suggested as the carrier rate in volunteer blood donors in the Philadelphia area\textsuperscript{14}.

The much higher frequency in the hepatitis patients as well as others with a high likelihood of contracting hepatitis, taken with its antigenic relation to Au(1), suggests that anti-Au(2) may detect additional specificities associated with hepatitis virus which are more widely distributed or more readily detected in human sera.

We thank Dr J. D. Nelson, Mr F. W. Russell, Dr A. Vierucci, Dr L. A. Potkonakt, Dr B. H. Marshall and Dr J. E. Prior for supplying valuable sera. This work was supported in part by grants from the US Public Health Service and by an appropriation from the Commonwealth of Pennsylvania.

\textbf{Cyril Levine}

\textbf{Baruch S. Blumberg}

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\textbf{Received November 11, 1968.}

\textsuperscript{1} Blumberg, B. S., \textit{Bull. NY Acad. Med.}, 40, 377 (1964).


\textsuperscript{10} Okochi, K., and Murakami, S., \textit{Vox Sang.} (in press).


\textsuperscript{14} Blumberg, B. S., Melartin, Liisa, Gerstley, Betty Jane S., and Werner, Barbara, \textit{J. Amer. Med. Assoc.}, 70, 541 (1965).


\textsuperscript{18} Blumberg, B. S., Alter, H. J., Riddell, Nancy, and Erlandson, Marion, \textit{Vox Sang.}, 9, 128 (1965).


New Specificity of Australia Antigen

The immunodiffusion laboratory at the Institute for Cancer Research frequently acts as a reference laboratory to test anti-Australia antigen sera for our colleagues in many parts of the world. Because Australia antigen is known to possess different antigenic specificities\(^1\)\(^-\)\(^4\), a panel was established which consisted of Australia antigen specimens selected from hepatitis and Down’s syndrome patients and from clinically normal residents of the Lau area in Malaita, British Solomon Islands. Sera from normal blood donors without Australia antigen were included as negative controls. All antisera received after August 1971 were tested against this panel to detect heterogeneity among both the antibodies tested and the antigens included in the panel. Immunodiffusion was performed in a seven-hole Ouchterlony pattern with the antiserum in the centre well and a positive Australia antigen control serum from a Pennsylvania Down’s syndrome patient in the top and bottom wells. The patterns were cut in a layer of 1.1% agarose in veronal buffer, \(pH\) 8.2, on glass lantern slides\(^5\)\(^,\)\(^6\).

Serum from a United States haemophilia patient (given by Dr Martin Goldfield) gave an unusual reaction with the sera in the panel. The antibody produced a line of complete identity between Australia antigen from Down’s syndrome and hepatitis patients from the United States, but gave a line of partial identity between these sera and the Australia antigen in the serum from Lau (Fig. 1). A spur indicated an additional antigenic specificity present on the Australia antigen from Down’s syndrome and hepatitis but absent in the sera from the Solomon Islands. This determinant will be called “f”, which appears to be distinct from other described determinants.

Sera from different parts of the world were tested with the anti-f serum (Table 1). All the 114 sera from the United States with Australia antigen contained the f determinant as opposed to the thirty-four sera from Lau containing Australia antigen, none of which had the f determinant. Sera both with and without determinant f were found in the samples from Bougainville, South America and the Far East.

Three independent lines of evidence indicate that the f determinant is not the same as the d and y specificities described by LeBouvier\(^4\) or Kim and Tilles\(^3\). First, d and y are differentially distributed from f (d and y both occur across the US population and appear to be mutually exclusive\(^4\); determinant f occurs in all the US sera tested). Second, the patterns of immunodiffusion reactions with specific anti-ad, anti-d and anti-y sera were compared to those of absorbed anti-f serum using a panel of sera containing Australia antigen (Table 2). The absorbed anti-f serum was produced by absorbing the haemophilia serum containing anti-f with a Lau serum lacking the f determinant. This absorbed anti-f reacted only with sera containing the f determinant. The anti-ad No. 1 and the anti-y were obtained from the National Heart and Lung Institute (NHLI). Anti-ad No. 1 was used at a dilution of 1:32, at which it reacts only with the d determinant among the samples from hepatitis and Down’s syndrome patients. The anti-d (LeB) and anti-y (LeB) were given to us by Dr G. LeBouvier. These and the anti-ad No. 2, from a US haemophilia patient, and the anti-y were used undiluted.

The pattern of reactions of the absorbed anti-f is different from that of either anti-ad, anti-d or anti-y, indicating that f is distinct from a, d or y (Table 2). There also appear to be anti-

<table>
<thead>
<tr>
<th>Area</th>
<th>Australia antigen positive</th>
<th>Australia antigen negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested</td>
<td>Determinant f</td>
</tr>
<tr>
<td>United States</td>
<td>195</td>
<td>114</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td>Down’s syndrome</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>Renal dialysis</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Leukaemia</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Blood donors</td>
<td>127</td>
<td>71</td>
</tr>
<tr>
<td>South America</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Peru (Cashinahua)</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Brazil</td>
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</tr>
<tr>
<td>Pacific Area</td>
<td>197</td>
<td>13</td>
</tr>
<tr>
<td>Lau, British Solomon</td>
<td>173</td>
<td>0</td>
</tr>
<tr>
<td>Islands</td>
<td>24</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 1 Occurrence of Specificity f in Australia Antigen from Different Geographic Areas
Fig. 1 Immunodiffusion pattern with spurs produced by the antiserum containing anti-f. The centre well contains whole anti-f serum; wells a and d contain Australia antigen from a US Down’s syndrome patient; wells b and f contain Australia antigen from Lau, British Solomon Islands; well e contains Australia antigen from Bougainville and well c Australia antigen from a US hepatitis patient. The sera from the US contain the f determinant.

bodies in the NHLI anti-y antiserum directed against a determinant, other than y, which occurs in the sera from Lau. This may be yet another new determinant.

Third, we mixed antisera to produce spurs which indicated non-identity between f and d and y (Fig. 2). Anti-y reacts only with the y antigen of a+y+f+ d−, and not with a+d+f+ y− (B). Absorbed anti-f reacts with the f of both a+d+f+ y− and a+y+f+ d− (C). A combination of these two (A) gives a partial line of identity which is the reaction of anti-f with a+d+f+ y− and a+y+f+ d−. The spur is the reaction of anti-y with the y determinant in a+y+f+ d−. This shows that f and y are not the same. The anti-ad (1:32) reacts with the d determinant of a+d+f+ y− but not with a+y+f+ d−. Absorbed anti-f reacts with the f of both a+d+f+ y− and a+y+f+ d− (F). In a combination of the last two (D), there is a line of partial identity which is the reaction of f with anti-f and a spur due to the anti-d reacting with a+d+f+ y−; this spur indicates that d and f are distinct.

Dr LeBouvier has given us antiserum to specificity x as well as serum containing Australia antigen with determinants a+y+x+ and a+d+x. These reagents were compared to
Table 2  Reactions of Australia Antigen from Different Sources with Specific Antisera

<table>
<thead>
<tr>
<th>Source</th>
<th>Anti-d Absorbed anti-ad Ab- (LeB)</th>
<th>Anti-x Anti-y Absorbed anti-ad Anti- (NHLI)</th>
<th>Anti-x Anti-y Absorbed anti-ad Anti- (NHLI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>82662 Solomon Islands</td>
<td>0 + + S 0 + +</td>
<td>0 + + 0 + +</td>
<td>0 + + 0 + +</td>
</tr>
<tr>
<td>82616 Solomon Islands</td>
<td>0 + + 0 + +</td>
<td>+ + 0 + +</td>
<td>+ + 0 + +</td>
</tr>
<tr>
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</tr>
<tr>
<td>82580 Solomon Islands</td>
<td>0 + + 0 + +</td>
<td>0 + + 0 + +</td>
<td>0 + + 0 + +</td>
</tr>
<tr>
<td>82613 Solomon Islands</td>
<td>0 + + 0 + +</td>
<td>0 + + 0 + +</td>
<td>0 + + 0 + +</td>
</tr>
<tr>
<td>82603 Solomon Islands</td>
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<tr>
<td>119491 Hepatitis</td>
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<td>+ + 0 + +</td>
<td>+ + 0 + +</td>
</tr>
<tr>
<td>119495 Hepatitis</td>
<td>+ 0 + + +</td>
<td>+ + 0 + +</td>
<td>+ + 0 + +</td>
</tr>
<tr>
<td>119497 Hepatitis</td>
<td>+ 0 + + +</td>
<td>+ + 0 + +</td>
<td>+ + 0 + +</td>
</tr>
<tr>
<td>117391 Down’s syndrome</td>
<td>+ 0 + + +</td>
<td>+ + 0 + +</td>
<td>+ + 0 + +</td>
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<tr>
<td>117425 Down’s syndrome</td>
<td>+ 0 + + +</td>
<td>+ + 0 + +</td>
<td>+ + 0 + +</td>
</tr>
<tr>
<td>117441 Down’s syndrome</td>
<td>+ 0 + + +</td>
<td>+ + 0 + +</td>
<td>+ + 0 + +</td>
</tr>
<tr>
<td>066474 Down’s syndrome</td>
<td>+ 0 + + +</td>
<td>+ + 0 + +</td>
<td>+ + 0 + +</td>
</tr>
</tbody>
</table>

* Diluted 1 : 32.  † S with NHLI serum only.
S = positive on stained plate only.

anti-f and Australia antigen with and without f. Absorbed anti-f reacted with both a+y+x+ and a+d+x- although it does not react with a+d+f- sera. Anti-ax, on the other hand, produces a spur between a+y+x+ and a+d+x- sera but no spur between a+y+x- and a+d+f-. These results indicate that the a+d+f- also carries x+. Therefore, f is not the same specificity as x.

Antiser to detect the b determinant contained in anti-Au(2) described by Levene and Blumberg are no longer available. Determinant f is highly unlikely to be the same as b, which was found in high frequency in sera which did not react at all with the anti-Australia antigen [anti-Au(1)] antiserum. The anti-f serum does not have this characteristic.

The determinant f has a distinct geographic distribution, which could be due to the association of f with an infectious agent with a restricted geographic range, to its relation to host genetic factors, or to both. More samples of Australia antigen collected in the United States are being examined to determine if Australia antigen which lacks f can be found and whether it is associated with a particular clinical diagnosis or exposure to agents from outside the United States.

We thank Dr James K. Roche of the National Heart and Lung Institute, National Institutes of Health; Dr Martin Goldfield of the New Jersey State Department of Health; and Dr George LeBouvier of the Department of Epidemiology and Public Health, Yale University, for the antisera used in this study. This work was supported by the US Public Health Service, the US National Institutes of Health, and the Commonwealth of Pennsylvania.

Note added in proof. While this work was in progress we became aware of an unpublished report of an additional Australia antigen determinant system called r and w (Bancroft, W. H., Mundon, F. K., and Russell, P. K., Journal of Immunology, in the press and personal communication from Dr W. Bancroft). Three antigen samples, kindly sent to us by Dr Bancroft, have been tested for f. Two of these that were scored positive for w were also positive for f, while the third was negative for both w and f but positive for r. Antibodies to w and r are required to establish conclusively the relationship between the three determinants. These antisera are not available at this time. Until the comparison can be completed, no statement can be made concerning this question.

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Received May 22, 1972.

Geographic Distribution of Australia Antigen Determinants d, y and w

Australia antigen (Au) has a group of antigenic determinants on its surface, which may occur in different combinations in the sera of people living in different geographical locations. Several of these determinants have been characterised and given alphabetical designations\(^5\). It has been reported that determinants d and y tend to be mutually exclusive\(^6\) although rarely they occur together (S. M. and S. B., unpublished). Determinants w and r also tend to occur in different samples rather than together and these two determinants have a strongly marked geographical distribution. Determinant w is common in the USA, South America, Europe and Africa, while r is common in Asia and the Pacific area\(^5\). We have tested 893 samples of Australia antigen from locations around the world for determinants d, y and w. Here we present these findings and speculate on factors which may contribute to the geographical distribution of Australia antigen subtypes.

The Australia antigen samples included in this study were all from apparently healthy asymptomatic carriers. They are part of the blood collection of the Division of Clinical Research at the Institute for Cancer Research and had previously been found to contain Australia antigen by immunodiffusion testing. Australia antigen determinants were identified using the standard seven hole immunodiffusion pattern cut in 1.1% agarose in pH 8.2 veronal buffer\(^6\). Specific typing sera were put in the centre well, control Australia antigen was put in the top and bottom wells, and test samples in the remaining four wells. Human anti-aw was reacted against an aw control antigen adjacent to the test samples. The formation of a line of identity between the control antigen and the test antigen was interpreted as indicating that the test antigen was aw. When a control antigen over the test antigen it was considered aw. The same principle was used in testing for determinant d with guinea pig anti-ad antisera (NIH V 801–502–058). A few sera which were known to be Australia antigen positive did not react with this serum at all. These will be considered d\(^–\). These and all other d\(^–\) sera were retested with a second anti-ad serum for confirmation of their d\(^–\) status. Absorbed chimpanzee anti-y and y control antigen were used to test for determinant y. Sera which were negative for both d and y were tested with an additional anti-ay serum.

The antigens have been grouped according to geographic origin of the sera and have been assigned to subtype categories suggested by Le Bouvier\(^6\) according to the presence of determinants d and y (Table 1). Subtype A consists of those antigens which lack both the d and y determinants. This group is designated A because by definition it must have determinant a which is defined as the universal or group determinant. The subtype categories have been further divided into w\(^+\) and w\(^–\). In all but four cases, determinants d and y were found to be mutually exclusive. These are not shown in Table 1. Two of these four exceptions were found on Bougainville and the other two were from people living in the United States who had travelled in Asia.

Australia antigen with determinant d is common in asymptomatic carriers in the United States, North Europe, Asia and Oceania, although subtype y does occur in these areas. All antigen samples from Japan carry the d determinant while none carry y. Antigen with the y determinant occurs almost to the exclusion of d in Africa and in Australian aborigines. Determinant y occurs frequently in India and in the Mediterranean area in Italy and Yugoslavia.

Virtually all antigens from Europe, the United States, Africa, India and Australia carry the w determinant. Antigen without w predominates in the New Hebrides, Micronesia, New Caledonia, Bougainville, Japan, Malaya and Thailand (Table 2). Both w\(^+\) and w\(^–\) occur in the

| Table 1 Distribution of w Determinant with Respect to Subtypes d, y and a in Asymptomatic Carriers from Various Geographical Locations |
|---------------------------------|-------|----------------|--------|----------------|--------|-------|
| Northern Europe                 |       |                 |        |                 |        |       |
| Belgium                         | 2     | 0               | 2      | 0               | 0      | 0     |
| Finland                         | 31    | 0               | 31     | 0               | 0      | 0     |
| Germany                        | 4     | 2               | 2      | 0               | 0      | 0     |
| Total                          | 37    | 35              | 0      | 2               | 0      | 0     |
| Mediterranean                   |       |                 |        |                 |        |       |
| Turk, Italy                    | 17    | 4               | 13     | 0               | 0      | 0     |
| Yugoslavia                     | 9     | 4               | 0      | 5               | 0      | 0     |
| Total                          | 26    | 8               | 0      | 18              | 0      | 0     |
| Africa                          |       |                 |        |                 |        |       |
| Ghana                          | 6     | 0               | 6      | 0               | 0      | 0     |
| Ivory Coast                    | 3     | 0               | 3      | 0               | 0      | 0     |
| Nigeria                        | 5     | 0               | 5      | 0               | 0      | 0     |
| South Africa (Africans)        | 2     | 1               | 1      | 0               | 0      | 0     |
| Senegal                        | 14    | 0               | 14     | 0               | 0      | 0     |
| Total                          | 30    | 1               | 29     | 0               | 0      | 0     |
| Asia                            |       |                 |        |                 |        |       |
| China                          | 4     | 3               | 1      | 0               | 0      | 0     |
| Japan                          | 91    | 25              | 66     | 0               | 0      | 0     |
| Philippines                    | 5     | 5               | 0      | 0               | 0      | 0     |
| Philippines (Cebu)             | 42    | 38              | 2      | 2               | 0      | 0     |
| Thailand                       | 3     | 2               | 0      | 0               | 0      | 0     |
| Total                          | 145   | 73              | 70     | 2               | 0      | 0     |
| Far East                        |       |                 |        |                 |        |       |
| India                          | 20    | 2               | 18     | 1               | 0      | 1     |
| Australia                      | 14    | 0               | 12     | 0               | 0      | 2     |
| Aborigine                      |       |                 |        |                 |        |       |
| Oceania                        |       |                 |        |                 |        |       |
| American Samoa                 | 3     | 0               | 2      | 1               | 0      | 0     |
| Bougainville                   |       |                 |        |                 |        |       |
| North                           | 34    | 0               | 34     | 0               | 0      | 0     |
| South Central                  | 172   | 17              | 155    | 42              | 35     | 1     |
| Total                          | 211   | 17              | 194    | 42              | 35     | 1     |
| French Polynesia               |       |                 |        |                 |        |       |
| Bora Bora                      | 3     | 2               | 1      | 0               | 0      | 0     |
| Tahiti                         | 13    | 6               | 7      | 0               | 0      | 0     |
| Tubai                          | 1     | 0               | 0      | 0               | 0      | 1     |
| Total                          | 17    | 8               | 8      | 0               | 0      | 1     |
| Malaita                        | 63    | 2               | 61     | 0               | 0      | 0     |
| New Caledonia                  |       |                 |        |                 |        |       |
| Konomi                         | 2     | 0               | 2      | 0               | 0      | 0     |
| La Foa                         | 0     | 0               | 0      | 5               | 1      | 0     |
| Nounue                         | 5     | 3               | 2      | 1               | 0      | 0     |
| Total                          | 13    | 3               | 7      | 3               | 0      | 0     |
| New Guinea                     |       |                 |        |                 |        |       |
| Various groups                 | 14    | 10              | 4      | 0               | 0      | 0     |
| New Hebrides                   |       |                 |        |                 |        |       |
| Aoba                           | 8     | 0               | 8      | 0               | 0      | 0     |
| Lamap                          | 16    | 1               | 15     | 0               | 0      | 0     |
| Norsup                         | 1     | 1               | 0      | 0               | 0      | 0     |
| Tanna                          | 17    | 0               | 17     | 0               | 0      | 0     |
| Vate                           | 27    | 1               | 26     | 0               | 0      | 0     |
| Total                          | 69    | 3               | 56     | 0               | 0      | 8     |
| Ponape                         | 5     | 0               | 5      | 0               | 0      | 0     |
| Rongelap                       | 6     | 1               | 5      | 0               | 0      | 0     |
| Utrik                          | 7     | 0               | 7      | 0               | 0      | 0     |
| Central and South America      |       |                 |        |                 |        |       |
| Brazil                         | 3     | 1               | 0      | 2               | 0      | 0     |
| Dominican Republic             | 19    | 11              | 8      | 0               | 0      | 0     |
| Jamaica                        | 2     | 0               | 2      | 0               | 0      | 0     |
| Peru (Cashinagua)              | 35    | 33              | 1      | 0               | 0      | 1     |
| Surinam                        | 11    | 8               | 2      | 0               | 0      | 3     |
| Yucatan                        | 3     | 3               | 0      | 0               | 0      | 0     |
| Total                          | 73    | 56              | 1      | 15              | 0      | 1     |
| North America                  |       |                 |        |                 |        |       |
| Frobisher Bay, Canada          | 2     | 0               | 2      | 0               | 0      | 0     |
| Philadelphia                   | 22    | 18              | 0      | 4               | 0      | 0     |
| Philadelphia (prisoners)       | 26    | 18              | 0      | 8               | 0      | 0     |
| Red Cross donors               | 90    | 76              | 1      | 13              | 0      | 0     |
| Total                          | 140   | 114             | 1      | 25              | 0      | 0     |

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population in China, the Philippines, French Polynesia, and New Guinea.

From these data and the data in Table 2, it seems that there are marked geographical separations of subtype. The basic northern European subtype is adw+ y- and the African subtype is ayw+ d-. In the Mediterranean area these subtypes both occur concurrently. The Asiatic type seems to be mostly adw+ y- with some ad+ wy-. Large areas of

Table 2 Distribution of Subtypes in Asymptomatic Persons Previously Described

<table>
<thead>
<tr>
<th>Location</th>
<th>Subtype D</th>
<th>Subtype Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tong*</td>
<td>30</td>
<td>11</td>
</tr>
<tr>
<td>Thailand†</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>Korea†</td>
<td>4†</td>
<td>3</td>
</tr>
<tr>
<td>New Guinea‡</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Singapore (China)§</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>Sweden‡</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>Germany†</td>
<td>38</td>
<td>2</td>
</tr>
<tr>
<td>Denmark‡</td>
<td>89</td>
<td>5</td>
</tr>
<tr>
<td>Israel†</td>
<td>13</td>
<td>47</td>
</tr>
<tr>
<td>Iran§</td>
<td>0</td>
<td>23</td>
</tr>
</tbody>
</table>

* 2 ayw, 20 adr; 1 probably adw.
† 3 ayw, 4 adr.
‡ A. Schober and R. Thomissen, personal communication.

Oceania are ad+ wy-. Japan is at a geographical interface with both adw+ y- and ad+ wy-. India and Australian aborigines are mostly ayw+ d- and Melanesia is in the interface between Australia (ayw+ d-) and the rest of Oceania (ad+ wy-).

Figure 1 shows the distribution of dominant subtypes from different parts of the world tested in this study.

Fig. 1 Distribution of Australia antigen subtypes. Each symbol represents the dominant subtype in that location. Triangles represent subtype d. Circles represent subtype y. Solid figures represent w+; open figures w-.

One hypothesis generated by this study is that the Australia antigen subtypes among asymptomatic carriers could be used as population markers and serve as an indication of human migrations. This would be more likely if women were included in the migration since when women carry the antigen they can transmit it to some of their children who may then become chronic asymptomatic carriers. When infants acquire Au from their mothers the subtype seems to be the same in both the mother and child. Daughters can then transmit the same subtype to their children and so on through many generations. This could maintain the subtype brought by the migrant population in a new environment. These introduced subtypes might not predominate since there are other mechanisms which produce carriers, but they should persist to some degree.

The subtype ayw+ d- is most common in Africa. In all areas tested where African slaves were introduced the ayw+ d- subtype occurs but in lower frequency than in Africa. These areas include the USA, Brazil, Jamaica, the Dominican Republic and Surinam. This is in contrast to northern Europe, Japan and Oceania where there are few people of African descent and little ay antigen. In the United States where about 10% (ref. 16) of the population is considered to be of African descent, 15% of the carriers are ay1, while in the Dominican Republic where 85% of the people have some African admixture, 42% of the Australia antigen is ayw+ d-. This hypothesis could be further tested by correlating subtype data with other biological data (blood and serum groups) and linguistic, archaeological and cultural information concerning human migrations. An Au subtype marker would be unique among known biological markers since it behaves as a unit and is passed from mother to child unaltered. In contrast, inheritable human biological markers are subject to Mendelian segregation and the resulting phenotypes can change rapidly from generation to generation.

Two United States chronic carriers were found to have w- antigen. They had both been in Vietnam and were presumed to have acquired their exotic subtype in Asia where w- is more common. We have tested a total of 318 Au samples from both patients and asymptomatic carriers in the United States and all except those two have had the w determinant. It is probable that some veterans of the Pacific theatre returned to the United States carrying ad+ wy- at the end of the Second World War. This subtype has not spread in the United States population to any measurable extent. This is compatible with the assumption that maternal transmission is the major mechanism for preserving an introduced subtype in a new environment.

South Central Bougainville is unusual in that there are seven different Au subtypes. It is unlikely that this diversity could be due to chance. The diversity could be related to the fact that Bougainville is located between Australia where the subtype is entirely ayw+ d- and Oceania where the complementary subtype ad+ wy- occurs frequently. In areas such as Bougainville two subtypes could meet and recombine by a mechanism similar to crossover which may occur in a doubly infected host, either human, animal, or arthropod. For example, if ayw+ d- and ad+ wy- agents coexist in the same host it is possible that they could recombine and yield four types of Australia antigen; the original ayw+ d- and ad+ wy-, as well as ay+ wd- and adw+ y- recombinants. All of these do in fact exist in Bougainville and have not been found together in other locations.

This work was supported by US Public Health Service grants and by an appropriation from the Commonwealth of Pennsylvania. We thank Drs J. Byrne, M. Goldfield, A. Prince and S. Hadziyannis for the antisera used in these studies, and all the investigators who contributed antigen samples.

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Silent Maternal Transmission of Australia Antigen


CHRONIC asymptomatic carriers of Australia antigen (Au, hepatitis B antigen, HBAg, HAA and so on) are found in human populations throughout the world and they constitute an important reservoir for the maintenance of the infectious agent. Here we provide evidence which, in combination with previous data, suggests that an important mechanism for the establishment of these carriers is through the ‘silent’ transmission (that is, without illness) of chronic infections from generation to generation through the maternal line.

There have been several studies related to mothers with Australia antigen and their children. We analysed 131 completed or partially completed families, from five groups [Cebu, Bougainville, Lau and Baeu speaking areas of Malaita, and Sardinian families from Turin]. There was a much higher frequency of Au in the offspring of matings in which the mothers were asymptomatic carriers of Au and the fathers were not, than in matings in which the fathers were carriers and the mothers were not (for all the families combined $P=0.55 \times 10^{-19}$ (ref. 2). Okochi and his colleagues found that of twelve children born to Japanese mothers who were asymptomatic carriers of Au, seven children became carriers within the first 6 months of life. Papaevangelou found that of eleven asymptomatic Greek mothers who had Au, two had babies who developed the antigen at 6 and 14 months, respectively. The antigen did not persist in these infants (G. Papaevangelou, personal communication). Turner *et al.* made a careful documentation of transmission of antigen from a mother with hepatitis to her infant. Subsequently, the infants of five mothers with Australia antigen positive hepatitis were studied and four were found to be carriers of the antigen without clinical signs of hepatitis.

In a larger study of mothers living in California who had Australia antigenaemia during pregnancy, or within two months of the birth of their children, Schweitzer and his colleagues found a significant incidence of vertical transmission. Of twenty-three women who had hepatitis, twelve gave birth to infants who developed Australia antigenaemia, some of which began, not at delivery, but within weeks or months afterwards. In each tested instance the babies had the same subtype as their mothers. The children did not have any evidence of hepatitis other than mild elevations of serum glutamic pyruvic transaminase (SGPT). Their development was normal although the antigenaemia has persisted for up to 23 months. Of fourteen mothers in this series who were chronic antigenaemic carriers one gave birth to a baby who became a chronic carrier. The frequency of transmission from asymptomatic mothers varies in different locations. In contrast to Japan (7/12), Greece (2/11) and California (1/14), in Denmark none of the babies of eighty-one carrier mothers became positive, and in Pakistan one out of eighteen infants of asymptomatic carrier mothers developed the antigen. These studies indicate that Au transmission from mothers with hepatitis is common and in some places maternal transmission from chronic carriers does occur.

The source of Au infection sometimes can be identified by immunological studies. The surface of the antigen is a composite of determinants which are immunologically distinct. Persons infected with material containing Au of a certain immunological combination, or subtypes, will develop those subtypes. The same subtype persists in both hepatitis patients and chronic carriers for as long as they have the antigen, with rare exceptions. We have used immunological subtyping to study relationships between Au carried by different members of the same family.

The sera used in this study were collected together with pedigrees in eighteen villages in south central Bougainville. They represented 85% of the healthy residents over the age of 2 yr. It was assumed that asymptomatic people with antigenaemia were chronic carriers. There was an Au carrier rate of 10.2% on Bougainville and family clustering had been observed in the initial testing of these sera. The distribution of Au had previously been subjected to segregation analysis which was compatible with autosomal recessive inheritance. The observed family clustering could also be explained by intrafamilial transmission of an infectious agent or maternal transmission as suggested by Turner *et al.* If this were true, then all members of a family should have the same subtype. The presence of multiple subtypes in the same family would rule against the family infection hypothesis. Sera for testing were selected from families with more than one antigen carrier so that a comparison between family members could be made. The subtyping was done by immunodiffusion in a standard seven-hole pattern cut in 1.1% agarose in veronal buffer, pH 8.2 (ref. 20). In preliminary testing for d and w the antigen was put in the centre well and a control Au which carried the determinant complementary to the antigen was put in the top and bottom wells. If a smooth line of identity was formed between the control antigen and the test antigen the test antigen was scored as positive for the determinant in question. If a spur was formed it was considered negative for the determinant. Absorbed antiserum was used to test for determinants y, w and d from a chimpanzee immunised with Au of subtype aywx was absorbed with adwx + y antigen, leaving anti-y in the antiserum. This was used with a y positive antigen control. Antigens giving a line of identity were considered positive; those giving no line, negative.

A few unusual sera carried neither d nor y. All such d — y — antigens were reacted with different dilutions of antisera and an additional anti-ad and anti-ay sera. All plates were dried and stained with azocarmine for better visualisation of precipitin lines.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Australia Antigen Subtypes Found in Eighteen Villages on Bougainville</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtype D</td>
<td>Subtype Y</td>
</tr>
<tr>
<td>Village</td>
<td>Au</td>
</tr>
<tr>
<td>Total</td>
<td>174</td>
</tr>
</tbody>
</table>

All possible combinations of determinants d, y and w except d + y + w — (Table 1) were found in this population. Of the 174 Au samples typed, two contained both d and y. This is a rare event and has not been reported before in normal people who have not received transfusions. These determinants seemed to be on the same particle (S. Mazzur and S. Burgert, personal communication).

Villages 2, 11, 13 and 16 did not contain families in which there were more than one member with Au. The data from families in the remaining villages which have more than one member with antigen are shown in Table 2. Of the thirty-two families tested there were eleven families in which two different combinations of antigens were segregating. Asymptomatic carriers living in the same household can have different subtypes of Australia antigen (Table 2). This means...
that these carriers have not been infected by each other nor from the same source. This rules out simple household infection or maternal transmission as an explanation for all the observed family clustering in these families.

A special case of family infection does, however, seem to occur. In families where the mother was an asymptomatic carrier, the twenty-two children who were carriers with a single exception had the same subtype as their mother. Among the twelve families where the mother was positive, one had mixed subtypes, while among the twenty families in which the mother did not have Au, ten families had antigen of different subtypes. These reflected the subtypes in the general population of the village. Among the six families in which the father was positive, there were mixed subtypes in four of the families. Children with Au from families where either the mother but not the father, or the father but not the mother, carried the antigen were compared using Fisher’s exact test (two-tailed). The difference in distribution was significant ($P=0.006$). When the families in which either parent was not available for testing were excluded from the analysis, the differences were still significant ($P=0.007$). The same test was used to compare the number of siblings of matching subtype in families with Au carrier and Au negative mothers. This difference was significant ($P=0.005$).

These data indicate that vertical transmission from asymptomatic mothers to their asymptomatic children occurs in this environment. Horizontal transmission with the production of carriers must also occur within this population since maternal transmission cannot account for all of the observed carriers.

Genetic predisposition to the chronic asymptomatic carrier state has been proposed by Blumberg20,21 and confirmed by Ceppellini1 on the basis of segregation analysis of the distribution of Australia antigen in several populations. Segregation analysis of the distribution of Au in the same Bougainville sera which were used in this study revealed the segregation pattern expected of autosomal recessive inheritance. The vertical transmission reported in this communication is compatible with the genetic hypothesis since the genetic analysis was applied also to matings in which neither parent was a carrier of Au. There would be no maternal effect in these families. There is not an occult maternal effect due to undetected levels of antigen in the mothers’ serum because the subtypes of the children do not, in general, match each other as they would in such a case.

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**Table 2** Distribution of Australia Antigen Subtypes in Families where More than One Member has the Antigen in the Blood

<table>
<thead>
<tr>
<th>Village</th>
<th>Father</th>
<th>Mother</th>
<th>Sibs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>w+d+y+</td>
<td>w+d+y+</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>w+d+y+</td>
<td>w+d+y+</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>w+d-y-</td>
<td>w+d-y-</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>w+d-y-</td>
<td>w+d-y-</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>w+d-y-</td>
<td>w+d-y-</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>w+d+y+</td>
<td>w+d+y+</td>
</tr>
<tr>
<td>12</td>
<td>NA</td>
<td>w+d-y+</td>
<td>w+d-y+</td>
</tr>
<tr>
<td>14</td>
<td>NA</td>
<td>w+d-y+</td>
<td>w+d-y+</td>
</tr>
<tr>
<td>16</td>
<td>NA</td>
<td>w+d-y+</td>
<td>w+d-y+</td>
</tr>
<tr>
<td>18</td>
<td>NA</td>
<td>w+d-y+</td>
<td>w+d-y+</td>
</tr>
</tbody>
</table>

* Indicates families with mixed subtypes. NA indicates not available.
† Insufficient amount to test for y.

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Women who become Au carriers by virtue of genetic susceptibility can be expected to transmit the antigen to some of their children whether or not the children are homozygous for the postulated recessive Au susceptibility gene. Thus, a new maternal transmission line can be established from a genetically susceptible woman whose own mother was not a carrier provided the susceptible woman is exposed to infection. The interaction of the genetic predisposition to the carrier state and maternal transmission could be an important source of Au carriers. Further, the interaction of these two mechanisms, one of which is dependent on the survival and reproduction of women who are carriers, would provide a very rapid amplification of the number of carriers of Au in an environment where such a carrier state is advantageous. Such a selective advantage has been suggested by Blumberg et al.²² Not all children of mothers who are Au carriers become carriers themselves. There are factors, either genetic or environmental or both, which influence the outcome of the maternal-child relationship. We suggest that other, perhaps undiscovered, human pathogens survive by the mechanisms...
described here and are usually expressed as disease when passed horizontally or after long incubation. Techniques developed through the study of Australia antigen as well as epidemiological patterns disclosed by these studies could serve as models for the investigation of these agents. It has been suggested that other infectious agents may exist that have properties similar to Australia antigen. These have been termed Icrons.

Silent maternal transmission with the production of asymptomatic carriers seems to provide a reliable mechanism for virus survival particularly in small populations where the number of new susceptible individuals is limited. The carrier state maintains the infectious agent over long periods of time and the maternal-child interaction provides exposure of new susceptibles. In contrast, many acute viral infections spread quickly, exhaust the availability of susceptible hosts and disappear from isolated populations.

This work was supported by United States Public Health Service grants from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania. We thank Drs J. Byrne, M. Goldfield, A. Prince and S. Hadziyannis for the antisera used in these studies as well as all who contributed antigen samples to the study.

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HEPATITIS B VIRUS

Sex Ratio
Sex Distribution of Australia Antigen

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The sex distribution of Australia antigen was determined in ten disease groups and 13 presumed normal populations tested in our laboratory. In 22 of these 23 independent studies, the frequency of Australia antigen was higher in males than in females ($P < .001$). The increased frequency in males was most marked in the lower age groups (less than 20 years). The overall ratio of Australia antigen in males to females is 1.58.

Men are not as hardy as women, and in nearly all environments men die at an earlier age. They also have a higher susceptibility than females to infection with a variety of organisms. Washburn et al estimated the male-female ratio for cases of neonatal septicemia and meningitis by collecting data from the literature (1930 to 1965) and from the case records of the Johns Hopkins Hospital, Baltimore (1896 to 1965). They concluded that, with the exception of organisms involved in prenatal infection, there was a higher frequency of infection in males than in females. Further, the difference was most marked in infants and even more striking in the post antibiotic age than before the widespread use of these drugs. They concluded from the latter that females were more amenable to therapy than males. Childs proposed a genetic hypothesis to explain the sex difference. He theorized that a gene involved with the synthesis of immunoglobulins is present on the X chromosome. In order to effect dosage compensation (ie, compensate for the presence of two X chromosomes in females and only one in the male), Lyon and others have proposed that there is random inactivation of the X chromosomes in the cells of females; this allows for heterozygosity in females which does not occur in males. Hence, if resistance to the effects of infection is present on the X chromosome, the female, because of her greater variability, could more effectively resist disease. Schlegel and Bellanti have suggested that a deficiency of glucose-6-phosphate dehydrogenase, which is X linked, may be one of the factors associated with susceptibility to infection, but this has been contested by Rodey et al.

Childs has also pointed out that in diseases characterized by an increased production of antibody (the so-called auto-immune or iso-immune diseases) there is a higher frequency of females than males involved (eg, disseminated lupus erythematosus, primary biliary cirrhosis, rheumatoid arthritis, thyroiditis).

Australia antigen was discovered in 1963, the association of Australia antigen with hepatitis was first found in 1966, and since then the association has been confirmed by many investigators. Further, the hypothesis that Australia antigen is a hepatitis virus has been supported by a variety of findings including transmission to...
Table 1.—Sex Ratio of Australia Antigen in Patients With Various Diseases and Normal Nonhospitalized Populations

<table>
<thead>
<tr>
<th>Disease</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Acute viral hepatitis²</td>
<td>112</td>
<td>40</td>
</tr>
<tr>
<td>Chronic hepatitis²</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Down's syndrome²</td>
<td>212</td>
<td>67</td>
</tr>
<tr>
<td>Other mentally retarded²</td>
<td>145</td>
<td>5</td>
</tr>
<tr>
<td>Leukemia³</td>
<td>173</td>
<td>22</td>
</tr>
<tr>
<td>Tuberculoid leprosy (India)⁴</td>
<td>396</td>
<td>45</td>
</tr>
<tr>
<td>Tuberculoid leprosy (India)⁵</td>
<td>451</td>
<td>32</td>
</tr>
<tr>
<td>Tuberculoid leprosy (Cebu)⁶</td>
<td>343</td>
<td>19</td>
</tr>
<tr>
<td>Tuberculoid leprosy (Cebu)⁷</td>
<td>281</td>
<td>8</td>
</tr>
<tr>
<td>Long-term dialysis (renal disease)⁸</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 42.3270 \quad P = .0025 \]

<table>
<thead>
<tr>
<th>Disease</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Acute viral hepatitis²</td>
<td>147</td>
<td>47</td>
</tr>
<tr>
<td>Chronic hepatitis²</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>Down's syndrome²</td>
<td>218</td>
<td>65</td>
</tr>
<tr>
<td>Other mentally retarded²</td>
<td>150</td>
<td>5</td>
</tr>
<tr>
<td>Leukemia³</td>
<td>171</td>
<td>22</td>
</tr>
<tr>
<td>Tuberculoid leprosy (India)⁴</td>
<td>404</td>
<td>45</td>
</tr>
<tr>
<td>Tuberculoid leprosy (India)⁵</td>
<td>451</td>
<td>32</td>
</tr>
<tr>
<td>Tuberculoid leprosy (Cebu)⁶</td>
<td>343</td>
<td>19</td>
</tr>
<tr>
<td>Tuberculoid leprosy (Cebu)⁷</td>
<td>281</td>
<td>8</td>
</tr>
<tr>
<td>Long-term dialysis (renal disease)⁸</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 47.6840 \quad P = .0058 \]

² All data are from our laboratory and have been published in the indicated references. Data without references have not been previously published.
³ Au(1) only detected in males.

Table 2.—Sex Ratio of Australia Antigen in Patients With Various Diseases and in Nonhospitalized Populations, by Age

<table>
<thead>
<tr>
<th>Group</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Lepromatous leprosy (Cebu)</td>
<td>85</td>
<td>24</td>
</tr>
<tr>
<td>Nonlepromatous leprosy (Cebu)</td>
<td>110</td>
<td>20</td>
</tr>
<tr>
<td>Tuberculoid leprosy (Cebu)</td>
<td>58</td>
<td>5</td>
</tr>
<tr>
<td>Bougainville</td>
<td>344</td>
<td>32</td>
</tr>
<tr>
<td>Polynesia</td>
<td>406</td>
<td>5</td>
</tr>
<tr>
<td>New Hebrides</td>
<td>370</td>
<td>42</td>
</tr>
<tr>
<td>New Caledonia</td>
<td>415</td>
<td>9</td>
</tr>
<tr>
<td>Down’s syndrome</td>
<td>67</td>
<td>32</td>
</tr>
</tbody>
</table>
humans and animals,7-9 by its virus-like appearance,10,11 identification in the nuclei of affected individuals,12,13 and in other ways. These findings have been reviewed (see for example14-19) and will not be elaborated further here. The use of the Australia antigen test (the test for Australia antigen in the peripheral blood) has permitted the investigation of the sex distribution in several diseases and populations. An increased frequency of Australia antigen in males was reported in our earlier publications,20-23 and additional data have now been assembled which confirm this.

Materials and Methods

The immunodiffusion method using the reinforcement pattern and staining with azo carmine was used for the detection of Australia antigen.23 Human and rabbit antisera (anti-Au) as described in previous publications were used in the studies.

Disease Groups.—The method of collection and description of these groups studied are given in the original publications referenced in Table 1. They are also described briefly here.

Acute and Chronic Active Viral Hepatitis.19-27—Physicians in the Philadelphia area were asked to submit sera of their patients diagnosed or suspected of having viral hepatitis. In most cases only a single sample was available from each patient. Serum values from patients with chronic active hepatitis were obtained from well documented cases at the Mayo Clinic.

Down’s Syndrome (Mongolism).—The majority of the Down’s syndrome patients housed in two large institutions in the Philadelphia area are included.5,19,26 They have been tested several times over the course of two to five years.

Leukemia.—These are transfused leukemia patients from the National Institutes of Health Clinical Center, Bethesda, Md, and from various hospitals in Philadelphia.23

Leprosy.—The lepromatous leprosy cases include nearly all the patients with this diagnosis in a large leprosarium on the island of Cebu in the Philippines. The tuberculoid leprosy patients were nearly all outpatients and represent about one third of the patients treated in the clinic at the time the study was performed.24 In the In-

Results

Australia antigen is more common in males than in females in all of the disease groups and in all of the populations except one (Table 1). The data were statistically analyzed in several ways:

1. A two-by-two contingency table was prepared for each disease group and the chi-square and probability value determined for each. There was a significant difference at the .01 level (or less) in four of the populations and two of the disease groups.

2. Since these are each independent series of observations, the P value for (a) all of the normal population groups, (b) all of the disease groups, and (c) the population and disease groups combined was determined using the formula

\[ X^2 = -2 \sum \log_i (P_i) \]

where the degrees of freedom is equal to the total number of P values combined. In this calculation P = .0025 for the disease group, P = .0058 for the normal population group, and P = .0001 for the combined group.

3. The sign test was used to compare groups a, b, and c just mentioned. In this, a positive sign was assigned to the population or disease group in which the males had a higher frequency than the females and a negative sign for the opposite. If there were no differences between males and females, then there would be about an equal number of positive and negative signs. But this was not true; the respective values for a, b, and c are +10, -0; +12, -1; and +22, -1; and the probability that these are due to chance are .001, .003, and <.001, respectively.

From all this we conclude that there is a significantly higher frequency of Australia antigen in males than in females.

In the populations in which age breakdown was possible (Table 2), the male-female ratio is higher in the younger (0 to 19 years of age) than the older (20+ years of age) group in every case but one. The overall P value by the sign test is .035.

Comment

This combined study confirms our earlier observations17-20 that in general there is a higher frequency of Australia antigen in males than in females. A review of sex frequency data in studies from other laboratories (A. Vierucci, MD, written communication, July 1970)23-26 provides further confirmation and emphasizes the universality of this relationship (Table 3). There could be some trivial explanations for the higher frequency in males; for example, in some of the populations it could be argued that male activities and exposures make them more likely than females to become infected. However, it is difficult to see how this would operate in all of the populations studied. For example, there is no major difference in activity between the institutionalized

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male and female patients with Down’s syndrome or lepromatous leprosy patients. Further, some unknown bias favoring the observed higher frequency in males could have been generated in some of the populations, but again it is hard to envision how this could happen in virtually all of the populations studied. These findings are consistent with the general observations of a higher frequency of infection in males than in females.

As noted in the introduction, Australia antigen is closely associated with viral hepatitis and may be the infectious agent or part of such an agent which causes a large proportion of the cases of hepatitis in man. Australia antigen is also associated with a group of chronic diseases (“Australia affinity group”) characterized by an impairment of immune function.11 These include Down’s syndrome, the lymphocytic leukemias, Hodgkin’s disease, lepromatous leprosy (but not tuberculous leprosy), and patients with chronic renal disease treated by repeated hemodialysis. In addition there are vast numbers of people living primarily in the tropics and Southeast Asia who are apparently asymptomatic carriers of Australia antigen. Family studies have been concluded in two of these populations12,13; on the basis of these it appears that susceptibility to persistent infection in these groups is inherited as a simple autosomal recessive trait controlled by a gene designated Au’. This has been supported by an independent study of Sardinians living in Turin.14

There are several observations relating to hepatitis and to the diseases of the Australia affinity group which may bear on the sex distribution. The frequency of lepromatous leprosy is greater in males than in females. The causative organism of leprosy (Hansen’s bacillus, Mycobacterium leprae) often localizes in the testes in patients with lepromatous leprosy whereas involvement of the ovaries is rare. Lepromatous males may become sterile.15 Similarly, male patients with Down’s syndrome are sterile while female patients are not.16 Males also predominate in number over females in leukemia.17 The testes are often invaded by a pathological process in male patients with acute leukemia while involvement of the ovary is less common.18 We found evidence of Australia antigen in the testes of one Down’s syndrome patient by an immunofluorescence technique.19 Australia antigen has been identified in the testes of a male cancer patient who had Australia antigen in his blood prior to his death.20

In addition to the genetic hypothesis proposed by Childs;2 these observations suggest a variety of testable hypotheses including the following: (1) Antibody to Australia antigen forms less frequently in males than in females; this may also be related to complex formation. (2) Cellular immune responses are less active in males than in females. (3) Lymphocytes of males are not as responsive to immunogenic stimuli as lymphocytes of females. Further, the age data suggest that these differences may change with increasing age. These hypotheses will be tested in forthcoming studies.

This work was supported in part by Public Health Service grants CA-06551, CA-06069, CA-08069, and RR-05539; and by an appropriation from the Commonwealth of Pennsylvania.
References

Comparison of Progeny of Mothers with and without Australia Antigen

By Kay Kukowski, W. Thomas London, Alton I. Sutnick, Max Kahn, and Baruch S. Blumberg

ABSTRACT

Blood specimens from 852 pregnant women and 699 newborns were tested for Australia antigen [Au(1)]. Au(1) was found in 7 of the women, but in none of the infants' cord blood, nor in 90 infants tested subsequently up to 3.5 months of age. Mothers with Au(1) had a longer gestation time and were younger than mothers without Au(1).

Australia antigen [Au(1)] is, or is located on, an infectious agent that can cause hepatitis in man (Blumberg et al., 1971). Persistent Au(1) is widely distributed throughout the world in people who are in apparent good health but who can be classified as carriers of Au(1) (Blumberg et al. 1971). Consequently, Australia antigen can occur in pregnant women who have hepatitis or in pregnant women who are clinically well. The effect of both such situations on the course of the pregnancy and on the infant may be of considerable importance. The purpose of this study is to investigate biological factors of the mothers who are “normal” chronic carriers of Australia antigen and their children.

The current study was carried out in North India. We have previously studied the prevalence of the Au(1) carrier state in the normal population in South India (2%, 5 of 251). From this we expected to find a higher frequency of positive females than in an equivalent study in the United States. Here we report 1) the frequency and age distribution of Au(1) in pregnant women from a North India population, 2) the effect of Au(1) in the mother on gestation time, birth weight, head circumference, length, and congenital anomalies of the baby, and 3) the failure to detect Au(1) in the umbilical venous (cord) blood of the offspring of these women.

1 From the Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111.
Materials and Methods

Blood was obtained from 852 pregnant women and 699 of their newborns at Holy Family Hospital (HFH), New Delhi, India, between March 1970 and September 1970. The women attending this hospital are largely from middle class families. Samples of blood were collected from all women in the course of prenatal care, and again at the time of delivery. Cord blood was obtained from infants at delivery. Maternal and cord blood were collected from 93.9% of the deliveries at HFH during the period from March 1970—August 1970. Blood was collected from as many infants as possible at 6 weeks and 3 months of age. These specimens were obtained by heel puncture and collected in microcentrifuge tubes (Table 1). All specimens were frozen and sent to The Institute for Cancer Research and tested for Australia antigen by the immunodiffusion technique (Blumberg et al. 1970).

Table 1
Frequency of Au(1) in North Indian Mother-Child Population

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Au(1)</th>
<th>% Au(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Sera</td>
<td>852</td>
<td>7</td>
<td>0.82%</td>
</tr>
<tr>
<td>Cord Sera</td>
<td>699</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Infant Sera</td>
<td>90</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*652 maternal-cord pairs were collected.
**Blood was obtained from 47 babies whose mothers were not tested. This includes the cord bloods from 6 of the 7 Au(1) mothers.
***Infant sera were obtained from infants age 2 days-3.5 months.

In order to determine the effects of Australia antigen on the baby, we recorded the findings on physical examination of the newborns. We noted congenital abnormalities, the babies' height, weight and head circumference. The gestational ages (measured from the date of last menstrual period), and the classification of newborn infants by using the method of Yerushalmy (1967) (Index of adequacy of intrauterine development) were determined. The gestational ages were compared with birth weight as recommended by Neligan et al. (1970). The statistical comparisons were made with the Mann-Whitney (1947) rank sum test and the Fisher (1934) exact \( 2 \times 2 \) test.
The data were obtained from data slips on the hospital and clinic records. The completeness of this information varied with the staff present (nurse, midwifery student, etc.). Occasionally a cord blood was not obtained from an infant on whom measurements were made. Data are available on more mothers than infants because some (including one with Au(1)) had not delivered before the termination of the study.

**Results**

Seven of 852 pregnant women had detectable Australia antigen. All seven were clinically well and specifically did not have hepatitis. Au(1) was absent in all of the 699 cord sera tested and in the sera of 90 infants tested from 2 days to 3.5 months of age (Table 1). The offspring of 6 of the 7 mothers with Au(1) were included. Detailed data are included in the appendix.

The mothers with Au(1) were compared with the mothers without Australia antigen [Au(0)] with respect to age, gravida and gestation time (Table 2). Significant differences (p < .05) were found for two of these comparisons. Mothers with Au(1) were younger than Au(0) mothers, and had a longer gestation time.

In order to determine whether the longer gestation time was related to the younger age, or specifically to Au(1), we matched all Au(0) mothers of the same gravida and age (±1 year) with the Au(1) mothers (Table 3). The younger Au(1) mothers had a significantly longer gestation time than did the younger Au(0) mothers (p = .009). No significant difference was found with the older mothers, but the numbers may be too small to demonstrate it.

The offspring of the Au(1) mothers were compared with the offspring of the Au(0) mothers with respect to length, weight and head circumference (Table 2). No significant differences were found in these parameters. The offspring of Au(1) mothers were all in Yerushalmi's Group V (Table 4). That is, they were in the category of lowest incidence of neonatal mortality. The 13 "congenital abnormalities" found in this study occurred only in the offspring of Au(0) mothers (see appendix). A pair of twins and a single birth had Down's syndrome.

One mother had viral hepatitis without Au(1) at the time of delivery. Her baby did not have Australia antigen at birth or subse-
Table 2
Comparison of Pregnancy and Progeny Characteristics of Mothers
with & without Australia Antigen
(More detailed data in Appendix)

<table>
<thead>
<tr>
<th>Group I</th>
<th>Group II</th>
<th>Criterion</th>
<th>N₁</th>
<th>N₂</th>
<th>U</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au(1)</td>
<td>Au(0)</td>
<td>Gestation time</td>
<td>7</td>
<td>198</td>
<td>395.0</td>
<td>-1.9651</td>
<td>0.025 longer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mother's age</td>
<td>7</td>
<td>788</td>
<td>1301.5</td>
<td>-2.4158</td>
<td>0.0079 younger</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mother's gravida</td>
<td>7</td>
<td>737</td>
<td>1911.5</td>
<td>-1.225</td>
<td>0.110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baby length</td>
<td>6</td>
<td>693</td>
<td>1996.5</td>
<td>-0.171</td>
<td>0.864</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baby head circ.</td>
<td>6</td>
<td>679</td>
<td>1363</td>
<td>-1.445</td>
<td>0.148</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baby weight</td>
<td>6</td>
<td>697</td>
<td>1772</td>
<td>-0.642</td>
<td>0.521</td>
</tr>
</tbody>
</table>

N₁ and N₂ are respectively, the number of Au(1) and Au(0) mothers. U is the Mann-Whitney Rank Sum statistic, and Z is the expression of this statistic as a standard normal deviate. The significance level, P, derives from the value of Z.
Progeny of Mothers with Australia Antigen

Table 3

Comparison of Gestation Times of Au(1) and Au(0) Mothers Matched for Age and Gravida

<table>
<thead>
<tr>
<th>Group 1, Au(1)</th>
<th>Group 2, Au(0)</th>
<th>N₁</th>
<th>N₂</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 yrs. grav. 1</td>
<td>19-21 yrs. grav. 1</td>
<td>4</td>
<td>20</td>
<td>0.00923</td>
</tr>
<tr>
<td>25 yrs. grav. 2</td>
<td>24-26 yrs. grav. 2</td>
<td>1</td>
<td>20</td>
<td>0.433</td>
</tr>
<tr>
<td>25 yrs. grav. 3</td>
<td>24-26 yrs. grav. 3</td>
<td>2</td>
<td>7</td>
<td>0.444</td>
</tr>
</tbody>
</table>

quentely but the infant was jaundiced and had a hemolytic anemia at birth. The baby died at the age of 6 weeks with acute gastroenteritis.

Discussion

This study has demonstrated that the carrier state for Au(1) in pregnant women is associated with measurable biological characteristics. The data apply only to apparently normal pregnant women who are chronic carriers of Au(1); pregnant women with viral hepatitis, with or without Australia antigen, were not included. The mothers with Au(1) were significantly younger and had longer gestation times than the Au(0) mothers. The prolongation of gestation time was independent of the age of the mother or the number of previous pregnancies. The finding that the Au(1) mothers were younger than the Au(0) pregnant women is consistent with the data from other populations.

Table 4

Birthweight and Gestation Time of Progeny of Au(1) and Au(0) Mothers According to the Yerushalmy Classification

<table>
<thead>
<tr>
<th>Group</th>
<th>Birth wt. (gm)</th>
<th>Gestation time (wk)</th>
<th>N</th>
<th>Au(1)</th>
<th>Au(0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1500 or less</td>
<td>More or less than 37</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>1501-2500</td>
<td>Less than 37</td>
<td>14</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>III</td>
<td>1501-2500</td>
<td>37 or more</td>
<td>24</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>IV</td>
<td>2501 or more</td>
<td>less than 37</td>
<td>18</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>V</td>
<td>2501 or more</td>
<td>37 or more</td>
<td>138</td>
<td>6</td>
<td>132</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td>196</td>
<td>6</td>
<td>190</td>
</tr>
</tbody>
</table>

P = .127
that Au(1) is more common in younger than older persons (Blumberg et al., 1966).

The babies of Au(1) mothers did not differ from babies of Au(0) mothers with respect to height, weight, and head circumference. By the Yerushalmy classification the infants of Au(1) mothers all fall into the group with the lowest risk of neonatal mortality. They also have no congenital abnormalities, but the numbers are too small to comment on an association. At any rate, it does not appear that Au(1) in the mothers is associated with congenital abnormalities in the progeny.

The failure to detect Au(1) in cord blood and specifically in the cord blood of Au(1) positive mothers is consistent with the reports of other workers. We have also tested the cord blood of the progeny of seven mothers with Au(1) from Philadelphia and none of the bloods contained Au(1) by immunodiffusion. Our 13 cases can be added to the 116 other reported cases in which cord bloods from mothers with Au(1) in their blood at delivery had been tested. Only three of these were found positive; two were from babies whose mothers had acute hepatitis with Au(1) during pregnancy and the other mother was a chronic carrier of Au(1) (London et al. 1969; Lyons et al. 1970; Schweitzer and Spears, 1970; Smithwick and Suat Cheng Go, 1970; Hawkes, 1970; Tolentino et al. 1971; Keyes et al. 1971; Skinhoj et al. 1972; Cossart et al. 1972; Marshall and Dudgeon, 1972; Nishioka, 1971; Peters, 1971).

The combined studies of infants from 129 mothers with Au(1) indicate that the presence of Au(1) in the cord blood is a rare occurrence. These findings suggest two hypotheses: 1) the placenta is an effective barrier to the transmission of Australia antigen, or 2) the antigen crosses the placenta but is inactivated or destroyed in the fetus or for other reasons is not detectable. The younger age and fewer children of Au(1) mothers raises the question of whether Au(1) impairs fertility. These findings might merely represent an effect of their shorter time in the childbearing age, but could also indicate that older women in the Au(1) group have been relatively infertile, so did not appear in this study. This should be tested in other populations and in follow-up studies.

ACKNOWLEDGMENTS

We wish to thank Dr. Blanche Fernandez and Mr. Thomas Rooney of the Holy Family Hospital, New Delhi, India for their assistance with
this project. This work was supported by U.S.P.H.S. grants CA-06551, CA-06927 and RR-05539 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania.

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APPENDIX

Characteristics of Mothers with [Au(1)] and without [Au(0)] Australia Antigen

<table>
<thead>
<tr>
<th>Mother's Age (years)</th>
<th>Gestation Time (weeks)</th>
<th>Gravida</th>
<th>Au(0)</th>
<th>Au(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Totals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>4</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>11</td>
<td>26</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>22</td>
<td>27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>43</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>32</td>
<td>29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>54</td>
<td>30</td>
<td>1</td>
<td>0</td>
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<td>23</td>
<td>68</td>
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<td>24</td>
<td>65</td>
<td>32</td>
<td>1</td>
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<tr>
<td>25</td>
<td>109</td>
<td>33</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>26</td>
<td>75</td>
<td>34</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>27</td>
<td>49</td>
<td>35</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>36</td>
<td>36</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>29</td>
<td>38</td>
<td>37</td>
<td>25</td>
<td>0</td>
</tr>
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Totals, 737 7
**Measurements of Babies of Australia Antigen Positive [Au(1)] and Negative [Au(0)] Mothers**

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<tr>
<th>Weight (ounces)</th>
<th>Length (inches) Au(0)</th>
<th>Length (inches) Au(1)</th>
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Congenital Abnormalities (Found only in Offspring of Au(1) Mothers)

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<th></th>
<th>Au(0)</th>
<th>Au(1)</th>
<th>Male</th>
<th>Female</th>
<th>Age</th>
<th>Gravida</th>
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<td>C053453 Extra finger left hand</td>
<td>+</td>
<td>0</td>
<td>x</td>
<td>26</td>
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<tr>
<td>C053678 Extra toe right foot</td>
<td>+</td>
<td>0</td>
<td>x</td>
<td>20</td>
<td>1</td>
<td></td>
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<tr>
<td>C053790 Club foot both legs</td>
<td>+</td>
<td>0</td>
<td>x</td>
<td>19</td>
<td>1</td>
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<td>C053900 Spina Bifida</td>
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<td>x</td>
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<td></td>
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<tr>
<td>C053924 Anencephalia (stillborn)</td>
<td>+</td>
<td>0</td>
<td>x</td>
<td>29</td>
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<tr>
<td>C053938 Rudimentary rt. ear</td>
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<td>0</td>
<td>x</td>
<td>35</td>
<td>4</td>
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<tr>
<td>C054175 Bilateral cleft palate with harelip</td>
<td>+</td>
<td>0</td>
<td>x</td>
<td>22</td>
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<td>C053023 Hydrocele bilateral</td>
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<td>C054804 Slight talip equino varies R. foot</td>
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<td>0</td>
<td>x</td>
<td>23</td>
<td>3</td>
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<tr>
<td>C053209 Slight talip L. foot posterior</td>
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<td>0</td>
<td>x</td>
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<td>C054766 Down's syndrome</td>
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<td>x</td>
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<td>C054193 Down's syndrome twins</td>
<td>+</td>
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<td>x</td>
<td>18</td>
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<td>192</td>
<td>+</td>
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<td>x</td>
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<tr>
<td>C054384 One testis undescended</td>
<td>+</td>
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<td>x</td>
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Hepatitis B Surface Antigen (Australia Antigen) in Parents and Sex Ratio of Offspring in a Greek Population

By J. E. Hesser, J. Economidou, and B. S. Blumberg

ABSTRACT

In data obtained from a Greek population, a significant increase in male livebirths was observed when either parent was positive for HB\textsubscript{e}Ag (Australia antigen), an antigen associated with the postulated hepatitis agent. Age, fertility, birth order, child-parent transmission, and a few socioeconomic items were some variables examined in relation to the increased sex ratio. The conclusion, based on the available evidence, is that HB\textsubscript{e}Ag in parents is possibly biologically associated with the sex of their progeny.

This paper presents observations from a study designed to test the hypothesis that there is an association between the sex ratio of offspring and the presence of Hepatitis B surface antigen (HB\textsubscript{e}Ag, Australia antigen, Au) in a parent.

HB\textsubscript{e}Ag is associated with an agent which can cause hepatitis and often occurs in the blood of people ill with hepatitis. It can occur as well in asymptomatic chronic carriers, and the blood from these people may cause hepatitis if transmitted to others. Many populations, especially those in tropical and Mediterranean areas, have a high prevalence of asymptomatic carriers of the antigen. The range is from 0.1% in Northern European populations to 20% in some Melanesian groups. The antigen may be detected by a simple immunodiffusion test as well as by more sensitive tests such as radioimmunoassay.

Studies in some populations have found a significant clustering of carriers in families (see, for example, Szmuness, 1973); significantly more HB\textsubscript{e}Ag-carrier children in families with positive mothers than in families with positive fathers or in families with both parents negative (Blumberg, 1972); and in one study, a significant identity of HB\textsubscript{e}Ag-subtypes between positive mothers and their positive children.

1 The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Penna. 19111.
2 Hellenic Red Cross, Blood Transfusion Center, 4 Alkiviadou, Athens, Greece.
but not between positive fathers and positive children (Mazzur et al. 1974). Vertical transmission and/or a genetic factor underlying susceptibility to infection have been proposed to account for these observations of both a maternal and a familial effect associated with \( \text{HB}_{\text{Ag}} \). The study described here was carried out to elucidate these familial and maternal effects.

The study was designed to test two hypotheses—one about fertility and one about sex ratio, of which the second is described at length below. That hypothesis was: the sex ratio of live births in matings with either the mother or the father positive for \( \text{HB}_{\text{Ag}} \) will be different from matings in which neither parent has \( \text{HB}_{\text{Ag}} \).

**METHODS**

Testing this hypothesis required the collection of reproductive histories and blood samples from a population known to have a high prevalence of \( \text{HB}_{\text{Ag}} \), on the assumption that adult individuals who are found to be carriers of the antigen have been so for many years. Previous studies have indicated that individuals who are carriers are likely to have had the antigen persistently (Blumberg et al. 1966), possibly from childhood. A prospective study on carrier parents in their childbearing years would be a way of testing the hypothesis which does not require making this assumption. Such a study was not considered warranted at this stage in our investigation.

A field study was done in Greece (where the prevalence of \( \text{HB}_{\text{Ag}} \) in some communities was known to be greater than 5%) in collaboration with the Hellenic Red Cross as part of their community donor screening program. Plati, a village in Macedonia, was the study site and was chosen for several reasons:

1. A screening project run by the Hellenic Red Cross indicated that there was an \( \text{HB}_{\text{Ag}} \) prevalence in the village of 8-10%. This was one of the highest frequencies found in their survey.
2. The village, with an estimated population of 1500 people, was of an appropriate size. Estimates based on a computer simulation run prior to the study through the courtesy of Dr. E. Lustbader of the Institute for Cancer Research, indicated that approximately 40 positive matings and 150 negative matings would be required to test the stated hypothesis.
3. The location of the village, transportation, and other facilities were logistically suitable for the study.
The field work was carried out between May and August of 1973. Laboratory work has been completed since September of 1973 and the laboratory and questionnaire data has been compiled in a computer storage facility at The Institute for Cancer Research. It is available for analysis through a general conversational program facility, designated CAPIA, devised by R. K. Stodola and E. Lustbader, for the Hewlett-Packard 2100A.

Plati is one of the many villages which was settled in Macedonia following the 1922 exchange of Christian Greek and Moslem Turkish populations between Greece and Turkey. One and a half million refugees had entered Greece from Asia Minor by 1928 (Pentzopoulos, 1963). The Plati population originates from refugees from the central region of Turkey, formerly called Cappadochia. From a population of about 1560 people (as recorded in the village census) blood samples were obtained from 1097 or about 70% of the population. The age distribution in the sample tested was about the same as that of the census population (Blumberg et al. 1974). Reproductive histories were obtained from 354 married women. These included genealogical and social items, and some attitudinal items known to relate to fertility, as well as information on all pregnancies, and on health. Blood was tested for the presence of HbAg, antibody to HbAg, and HbAg specificities, as well as for a large number of polymorphisms. The results of these other studies are to be described in detail elsewhere.

Reverse passive hemagglutination (Hepanosticon(R): Organon), and immunodiffusion (ID) (Blumberg et al. 1965) tests were run on the entire sample (n = 1097), and radioimmunoassay (RIA) (by a modification of Purcell et al. 1973) on 1011. HbAg was detected, as indicated by the result of one or more of these tests, in the sera of 123 individuals (11.3% of the total tested population). The distribution of HbAg by age and sex, for the tested population, is given in Table 1.

Testing and evaluating the stated hypothesis proceeded as follows:

1. Mating types were grouped according to the presence or absence of HbAg in the parents.
2. For each group, the age of mothers, the number and sex of livebirths, and the number of abortions was determined, and the sex ratio of livebirths calculated.
3. The age of mothers and fertility for each of the groups was compared.
Table 1

Distribution of HB$_x$Ag by Age and Sex. HB$_x$Ag was scored as present if the serum was positive by one or more of the following tests: Immunodiffusion, Radioimmunoassay, or Reverse Passive Hemagglutination. A question mark indicates an ambiguous result. These were not included in the final calculation.

| Age in Years | No. Males | | | No. Females | | |
|--------------|-----------|----------------|----------------|----------------|----------------|
|              | HB$_x$Ag(+) | HB$_x$Ag(0) | Total | % HB$_x$Ag(+) | HB$_x$Ag(+) | HB$_x$Ag(0) | Total | % HB$_x$Ag(+) |
| 0-9          | 9          | 94            | 103   | 8.7           | 16            | 84           | 100   | 16.0          |
| 10-19        | 13 + 2?    | 84            | 97    | 13.4          | 18 + 1?       | 66           | 84    | 21.0          |
| 20-29        | 5          | 24            | 29    | 17.0          | 6 + 3?        | 59           | 67    | 8.9           |
| 30-39        | 9          | 64            | 73    | 12.3          | 14            | 79           | 93    | 15.0          |
| 40-49        | 9          | 60            | 69    | 13.0          | 7             | 52           | 59    | 11.0          |
| 50-59        | 5          | 40            | 45    | 11.0          | 5             | 59           | 64    | 7.8           |
| 60-69        | 4 + 1?     | 52            | 55    | 5.5           | 2             | 62           | 64    | 3.0           |
| 70-79        | 1          | 26            | 25    | 4.0           | 0             | 26           | 26    | 0             |
| 80+          | 0          | 8             | 8     | 0             | 0             | 3            | 3     | 0             |
| unknown      | 0          | 18            | 18    | 0             | 0             | 7            | 7     | 0             |
| Totals       | 55 + 3?    | 470           | 528   | 10.5%         | 68 + 4?       | 497          | 569   | 12.0%         |

Total 123/1097 = 11.3% HB$_x$Ag(+)
4. A test of the hypothesis of no difference in the sex ratio between the groups was made and rejected.

5. A test of the hypothesis of no difference in the sex ratio between the groups was made, with parents' sex disregarded, and mothers grouped into age cohorts. A maternal age effect was discerned.

6. Several explanations which might account for the observation were tested and rejected.

**Results**

Mating types distinguished by the presence or absence of HBsAg in parents were grouped as follows:

1. Twenty-seven matings in which the mother was positive and the father was either negative (+ x 0) or untested (+ x ?). In eight in which only the mother had been tested, it was assumed that the father was most likely to be HBsAg negative, given the frequency of HBsAg in the total population. In the calculations the (+ x 0) and the (+ x ?) matings were grouped together.

2. Twenty matings in which the mother was negative and the father was positive (0 x +).

3. One hundred seventy-two matings with both parents found to be negative for HBsAg (0 x 0).

4. Eighty-three matings in which only the mother had been tested and was HBsAg (0 x ?). Since 10% of these presumably could be (0 x +), they were not included in the analyses, although these data are included in the tables.

5. Four matings in which both the mother and father were positive for HBsAg (+ x +). Because these matings were considered to be possibly biologically different from matings with one parent positive, these were separated from the other matings and calculations were done both with, and without, their inclusion in the group with one parent HBsAg (+).

There were 48 matings not included in the analyses. These were matings in which only the father or the mother had been tested and was negative, or had ambiguous test results, and from whom we did not obtain complete histories.

Mean values for age of mothers, age at marriage, and the number and sex of livebirths, and the number of abortions reported by them, was determined for each of the mating groups (+ x 0), (0 x +), (+ x +), (0 x 0), and (0 x ?) (Table 2).
Table 2

Mean Values for Mother's Age, Mother's Age at Marriage, Number of Livebirths, Induced and Spontaneous Abortions, for Matings of Parents with and without HBsAg (Mo = Mother, Fa = Father)

<table>
<thead>
<tr>
<th>Mating</th>
<th>Mo(+) × Fa(o) (n = 27)</th>
<th>Mo(o) × Fa(+) (n = 20)</th>
<th>Mo(+) × Fa(+) (n = 4)</th>
<th>Mo(o) × Fa(o) (n = 172)</th>
<th>Mo(o) × Fa(?) (n = 83)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
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<td>41.3</td>
<td>35.0</td>
<td>44.7</td>
<td>51.6</td>
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<tr>
<td>Age at marriage</td>
<td>28.1</td>
<td>26.5</td>
<td>19.0</td>
<td>26.7</td>
<td>21.0</td>
</tr>
<tr>
<td>No. of induced abortions*</td>
<td>2.63</td>
<td>1.75</td>
<td>2</td>
<td>2.4</td>
<td>3.16</td>
</tr>
<tr>
<td>No. of spontaneous abortions*</td>
<td>1.30</td>
<td>1.14</td>
<td>0</td>
<td>1.59</td>
<td>1.24</td>
</tr>
</tbody>
</table>

* n's for number of abortions represent only those women of the total who experienced these events. All others experienced none.
Hepatitis B Surface Antigen

The slight differences in the mean age of mothers in the groups were not statistically significant \((0 \times ?)\) matings were not included. This was determined by a test of the null hypothesis of no differences in the mean ages using Scheffé F projections (Miller, 1967).

Scheffé F projections were also calculated to test the null hypothesis of no difference between groups for the mean values for each of the fertility indicators (e.g. the number of livebirths and abortions). No significant fertility differences could be detected in relation to the presence or absence of HB,Ag in parents. The sex ratio (defined as the number of male offspring/the number of female offspring \(\times 100\)) of livebirths was calculated for each of the groups (Table 3). For matings with the mother HB,Ag positive, the sex ratio of livebirths is 175; with the father HB,Ag(+) it is 200; with both parents positive it is 100; for matings with both parents HB,Ag(0) it is 113. The difference in the number of male and female livebirths between matings with HB,Ag(0) parents and those with one parent HB,Ag(+) is statistically significant \((p = .018)\) (Table 4). Inclusion of the \((+ \times +)\) matings with the other matings with an HB,Ag(+) parent does not appreciably change the significance of this result \((p = .024)\). The observed relation between HB,Ag in parents and an increased sex ratio in their offspring is apparently independent of the sex of the affected parent.

A subsequent analysis of the sex ratio results disregarded the sex of the affected parent but took into account the proportion of males born to different age cohorts of mothers (ages 20-39, 40-59 and 60+) (Table 5). A significant difference in the proportion of males born to (+) mothers in the youngest cohort (20-39) was found; this was not true in the older cohorts. The effect observed in the youngest cohort of mothers can account for the effect observed in the total sample. One would expect to observe the same effect in all age cohorts unless there was some special characteristic of the younger women (possibly they acquired their antigenemia at a different age or in a different way than the older mothers).

Major differences between the mating groups, or between age cohorts, could not be determined when groups are compared for occupation, education, or birth place, or for differences in prenatal mortality. Positive parents did have a greater proportion of (+) offspring (23%) than did HB,Ag negative parents (12%). However, there were not significantly more (+) male offspring in any of the
### Table 3

**Sex of Live Births Reported for Matings of Parents with and without HBsAg (Mo = Mother, Fa = Father, NT = Not Tested)**

<table>
<thead>
<tr>
<th>Matings</th>
<th>No. Males</th>
<th>No. Females</th>
<th>M/F ratio</th>
<th>No. Matings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo (+) × Fa (0) or NT</td>
<td>49</td>
<td>28</td>
<td>175.0</td>
<td>27</td>
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<tr>
<td>Mo (0) × Fa (+)</td>
<td>36</td>
<td>18</td>
<td>200.0</td>
<td>20</td>
</tr>
<tr>
<td><strong>Subtotal, One Parent HBsAg (+)</strong></td>
<td><strong>85</strong></td>
<td><strong>46</strong></td>
<td><strong>185</strong></td>
<td><strong>47</strong></td>
</tr>
<tr>
<td>Mo (+) × Fa (+)</td>
<td>5</td>
<td>5</td>
<td>100.0</td>
<td>4</td>
</tr>
<tr>
<td><strong>Subtotal, HBsAg (+) Parents</strong></td>
<td><strong>90</strong></td>
<td><strong>51</strong></td>
<td><strong>177</strong></td>
<td><strong>51</strong></td>
</tr>
<tr>
<td>Mo (0) × Fa (0)</td>
<td>287</td>
<td>255</td>
<td>113</td>
<td>172</td>
</tr>
<tr>
<td>Mo (0) × Fa (NT)</td>
<td>135</td>
<td>135</td>
<td>100.0</td>
<td>83</td>
</tr>
<tr>
<td><strong>Total, all matings</strong></td>
<td><strong>509</strong></td>
<td><strong>496</strong></td>
<td><strong>103</strong></td>
<td><strong>306</strong></td>
</tr>
</tbody>
</table>

Three cohorts of (+) matings. (Results on the distribution of carriers within families will be discussed in a forthcoming paper.)

The relation to an altered sex ratio in a human population of an agent (HBsAg) closely related to infectious disease has, to the authors' knowledge, been previously reported only twice (Mazzur and Watson, [1970]).

### Table 4

**Chi-square Test (with Yates Correction*) for Significance of Difference in Number of Males and Female Livebirths Reported for Matings of Parents with and without HBsAg**

<table>
<thead>
<tr>
<th>Compared with:</th>
<th>No. Males</th>
<th>No. Females</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both parents HBsAg(0</td>
<td>287</td>
<td>255</td>
<td></td>
</tr>
<tr>
<td>1. Mo (+) × Fa (0)</td>
<td>49</td>
<td>28</td>
<td><strong>.10</strong></td>
</tr>
<tr>
<td>2. Fa (+) × Mo (0)</td>
<td>36</td>
<td>18</td>
<td><strong>.07</strong></td>
</tr>
<tr>
<td>1 and 2</td>
<td>85</td>
<td>46</td>
<td><strong>.018</strong></td>
</tr>
<tr>
<td>3. Fa (+) × Mo (+)</td>
<td>5</td>
<td>5</td>
<td><strong>.024</strong></td>
</tr>
<tr>
<td>1, 2, and 3</td>
<td>90</td>
<td>51</td>
<td><strong>.47</strong></td>
</tr>
<tr>
<td>4. M (0) × Fa (0)</td>
<td>135</td>
<td>135</td>
<td></td>
</tr>
</tbody>
</table>

* Yates corrected Chi-square yields a conservative estimate of p.
Table 5

Sex Ratio of Offspring of Different Maternal Age Cohorts, for Matings of Parents with and without HBsAg. p Value Is for a Chi-Square Test Comparing the Numbers of Males and Females within Each Age Cohort, between Matings of Parents with and without HBsAg (0 × ? Not Included)

<table>
<thead>
<tr>
<th>Mothers Age</th>
<th>Mating Type (+ × 0) and (+ × +)</th>
<th>(0 × 0)</th>
<th>(0 × ?)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>η η</td>
<td>φ φ</td>
<td>δ δ</td>
</tr>
<tr>
<td>20-39</td>
<td>42</td>
<td>19</td>
<td>80</td>
</tr>
<tr>
<td>40-59</td>
<td>39</td>
<td>23</td>
<td>122</td>
</tr>
<tr>
<td>60+</td>
<td>9</td>
<td>9</td>
<td>85</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>51</td>
<td>287</td>
</tr>
</tbody>
</table>
1975; Robertson and Sheard, 1973). An association of HBsAg in parents with the sex of their progeny is reported here we believe for the first time. It would be premature at this time to offer a biological explanation for the observations made, especially since very little is known about factors determining the sex ratio of offspring. If our observations are substantiated by further investigations, they can stimulate new studies designed to elucidate the biological mechanisms involved in sex ratio determinations.

ACKNOWLEDGMENT

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LITERATURE CITED


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Hepatitis B Surface Antigen


Hepatitis B Virus and Sex Ratio of Offspring

The response to hepatitis B virus infection in parents is related to the sex ratio of their children.

Jean S. Drew, W. Thomas London, Edward D. Lustbader
Jana E. Hesser, Baruch S. Blumberg

On a genetic basis, one would expect the ratio of males to females at birth to be one. In humans, this is not the case. In most human populations there is an excess of males born, and the secondary sex ratio (the number of males per 100 females at birth) falls within a range of 104 to 107 (1). It is not known whether this phenomenon is due to a high sex ratio at conception, or to a high mortality of female zygotes in the earliest stage of pregnancy (that is, before pregnancy is recognized). Observations on spontaneous abortions, stillbirths, and postnatal mortality show that male mortality exceeds female by anywhere from 40 to 145 percent (2). Hence, by the time reproductive age is reached the sex ratio is closer to 100 (3, 4). It is probably this ratio that is stabilized by natural selection (3-5).

There are geographic and racial differences in secondary sex ratio. For example, black populations have consistently lower sex ratios at birth than non-black populations (1, 6). However, significant deviations from the range of 104 to 107 are infrequent in countries with adequate ascertainment (1, 7). Temporal variations in sex ratio have been observed in relation to environmental factors, such as wars (8) and seasons (9); but sustained changes in secondary sex ratio probably occur too slowly to detect (3-5).

It has been suggested that a cultural preference for offspring of one sex could affect sex ratio. This has been seen in regard to postnatal sex ratios in societies in which a preference for one sex results in infanticide of the other (10). Birth control alone cannot affect secondary sex ratio, however, because the probability of a male at each birth is independent of the sex of previous births. In contrast if couples within a population vary in their inherent or biological probability of having a male, sex preferences, expressed in terms of effective birth control, could affect the secondary sex ratio (4, 11).

Intrapopulation variations in sex ratio have been attributed to genetic and environmental as well as racial differences (12, 13). Sanghvi (12) found an association of the ABO blood groups of mothers and their children with secondary sex ratio. In both Bombay and New York, the sex ratio of “O” infants born to “O” mothers was significantly higher than that of “A” infants born to “A” mothers. Jackson et al. (13) found a relation between the Xg® blood group system and sex ratio. Sex ratio was significantly higher among offspring of Xg®+ father × Xg®- mother matings than among those of all other Xg® combinations. They suggested that sensitization of Xg®- mothers by Xg®+ female fetuses might lead to a preferential loss of such embryos. In a recent analysis of data from the United States, Erickson examined the simultaneous effects of mother’s age, father’s age, mother’s race, birth order, legitimacy, and paternal education on sex ratio. The only significant effect detected was a negative association between birth order and secondary sex ratio (14).

A relation between sex ratio of offspring and an infectious agent has been observed in some species of Drosophila, in which maternal infection with small iroplastic spheroplasts, presumably treponematoids, is associated with a reduction in male (specifically single-X chromosome) offspring (15, 16). This phenomenon appears to be the consequence of a disturbance in the development of male zygotes, resulting in 50 percent mortality (15-17).

A relation between sex ratio of offspring and an infectious agent in a human population was reported in 1975. Hesser et al. (18, 19) found a significant association between sex ratio of offspring and the responses of parents to infection with hepatitis B virus (HBV) in a Greek population. In this article, we report our investigation of the relationship between HBV and sex ratio, and suggest a possible explanation for this phenomenon.

Host Responses to HBV Infection

Hepatitis B surface antigen (Australia antigen; HBsAg) is in the outer coat of the Dane particle, the presumed virion of HBV. Three types of particles (spherical, rod-shaped, and Dane particles), all with HBsAg on their surfaces, may be found in the blood of patients infected with HBV. When infected, an individual may respond by developing acute hepatitis, which is demonstrated by the transient appearance of HBsAg in his blood, elevated serum transaminase, and sometimes jaundice. Infection is followed by the development of antibody against HBsAg (anti-HBs) and the resolution of the viremia and clinical symptoms. In some cases, however, people become chronically infected with HBV, with little evidence of liver damage and few clinical symptoms. Many of these people do not develop anti-HBs, and they may be HBsAg+ for years (20). Such people are termed “chronic carriers” of HBV.

They may infect other uninfected persons (by transfusion, for example) and apparently are at increased risk of developing certain chronic liver diseases such as chronic aggressive and chronic persistent hepatitis, postnecrotic cirrhosis, and primary hepatocellular carcinoma (21).

HBV and Sex Ratio at Birth

HBV can be transmitted from mothers to offspring in the perinatal period, that is, in utero, during, or soon after birth. Infection early in life with HBV appears to increase the likelihood of becoming a chronic carrier of the virus (22), and vertical transmission of HBV from mother to child has been implicated as a major mechanism for the establishment of carriers in at least one population (23).

Transmission from father to child has not
been studied extensively but appears to be less common (24).

An association between hepatitis infection and sex ratio at birth was previously suggested by Robertson and Sheard, who found a relation between an outbreak of hepatitis (of unknown etiology) and a subsequent decrease in male live births (25). Mazzur and Watson described a high proportion of males among siblings of HBsAg carriers in Greece (26) and a subsequent decrease in male live births (25). Robertson and Sheard observed a high proportion of males among siblings of HBsAg carriers in Greece (26). In 1973, we studied differences in sex ratio associated with HBsAg in parents from the Greek village of Plati in southern Macedonia (27). When either parent was HBsAg(+), there was a significantly higher sex ratio of live births than when both parents were HBsAg(-), but at least one parent was HBsAg(-):anti-HBs(-) (18, 19, 28).

The original analysis of the Plati data considered only the presence of HBsAg. We subsequently hypothesized that anti-HBs would be associated with a sex ratio alteration opposite in direction to that associated with HBsAg. In this article, we present an analysis of the Plati data, including the data on anti-HBs as well as other factors thought to affect secondary sex ratio such as parental age, number of years of marriage, and order of birth and pregnancy. The conclusions from the Plati data are compared to observations from other studies showing an interaction of HBV with sex, and a hypothesis explaining the interaction is proposed.

### Results from Plati

Serums were obtained from 326 wives and 248 husbands, representing a total of 589 families. Reproductive histories, including the number and sex of live births and the number of abortions, were obtained from all the women and from those men whose wives were not available for the study. The sex ratio of the 1558 people (including parents and children) ascertained in this community was 111. This may seem high, but it is not unusual for Greece and is not significantly different from sex ratios encountered in most human populations. The normal range of sex ratios (from 104 to 107) falls within the 95 percent confidence limits of the ratio in Plati (from 100.4 to 122.7) (6).

Complete information, with respect to HBV serology and sex of all live births, was available from 323 couples. These couples were categorized according to immune responses to HBV, as defined by the presence or absence of HBsAg and anti-HBs in the serum of each parent (29). The sex ratio of offspring of 33 couples in which either parent was HBsAg(+):anti-HBs(-) was compared to that of 183 couples in which both parents were HBsAg(-). Among the 183 couples, the offspring of 29 couples in which both parents were anti-HBs(-) were compared to those of 154 in which either parent was anti-HBs(+). There were 16 couples in which neither parent was HBsAg(+):anti-HBs(-), but at least one parent was HBsAg(+):anti-HBs(+). These 16 couples are included in Table 1 but were excluded from further analysis (30).

<table>
<thead>
<tr>
<th>Parents' response to HBV</th>
<th>Couples (No.)</th>
<th>Live births*</th>
<th>Sex ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Either parent HBsAg(+) : anti-HBs(-)</td>
<td>33</td>
<td>60 (1.82 ± .22)</td>
<td>24 (0.73 ± .11)</td>
</tr>
<tr>
<td>Both parents HBsAg(-) : anti-HBs(-)</td>
<td>29</td>
<td>51 (1.76 ± .24)</td>
<td>35 (1.21 ± .23)</td>
</tr>
<tr>
<td>Both parents HBsAg(-) : either parent anti-HBs(+)</td>
<td>154</td>
<td>241 (1.56 ± .10)</td>
<td>222 (1.44 ± .10)</td>
</tr>
<tr>
<td>Neither parent HBsAg(+) : anti-HBs(-) and either parent HBsAg(+):anti-HBs(+)</td>
<td>16</td>
<td>25 (1.56 ± .39)</td>
<td>27 (1.69 ± .42)</td>
</tr>
</tbody>
</table>

- The numbers in parentheses indicate the number of live births per couple (mean ± S.E.). The numbers in parentheses indicate the 95 percent confidence limits in the sex ratio.

- The numbers in parentheses indicate the 95 percent confidence limits in the sex ratio.

![Least-squares fits for the regressions of sons and daughters as a function of the number of years of marriage.](image)

**Fig. 1.** Least-squares fits for the regressions of sons and daughters as a function of the number of years of marriage. By analysis of covariance (56), the number of sons per years of marriage is significantly higher \( P < .02 \) and number of daughters significantly lower \( P < .02 \) for HBsAg(+) as compared to anti-HBs(+) couples.
HBV in parents and sex ratio of offspring was further characterized by comparing the mean numbers of sons and daughters born per family to each category of parents. Table 1 shows that HBsAg(+) couples had similar numbers of sons but significantly fewer daughters per family as compared to anti-HBs(+) couples ($P = .0007$) (29).

Some sex ratio effects might be explained by other factors such as parental ages, number of years married, birth order, and fetal loss. Age is of particular importance because differences in the frequencies of HBsAg and anti-HBs by age are well documented (33). We tested the hypothesis that these factors could account for our observations in several ways. First, we compared the sex ratios and mean numbers of sons and daughters per family for only couples married more than 15 years. In this way we could examine presumably "completed" families and therefore control for effects of parental ages and birth order. This subgroup included 18 HBsAg(+), 17 HBsAg(-):anti-HBs(–), and 97 anti-HBs(+) couples (for a total of 132 families, more than 60 percent of the total sample). Again, HBsAg(+) couples had a significantly higher sex ratio ($P = .002$), the same number of sons, and fewer daughters ($P = .002$) than anti-HBs(+) couples. We then compared by analysis of covariance the least-squares fits for the regressions of sons and daughters as a function of number of years married. HBsAg(+) couples had significantly more sons ($P < .02$) and fewer daughters ($P < .02$) per number of years of marriage than did anti-HBs(+) couples (Fig. 1) (34). The fact that in HBsAg(+) couples total number of sons per family was similar, but that there were more sons per number of years married than in anti-HBs(+) couples, suggests that the HBsAg(+) couples had sons earlier in marriage than the anti-HBs(+) couples.

Figure 2 presents the proportions of males born to HBsAg(+) compared to the number born to anti-HBs(+) couples; these data take into account parental and maternal ages and pregnancy and birth order (35). The HBsAg(+) couples had consistently higher proportions of male live births than anti-HBs(+) couples, regardless of the effects of the other four factors. These overall differences, as compared by the Mantel-Haenszel method for stratified analysis (36), were significant (see legend to Fig. 2).

We considered two additional questions. (i) Although parental ages and pregnancy and birth orders individually did not account for the relation between response to HBV in parents and sex ratio of offspring, could these factors account for the phenomenon if considered simultaneously? (ii) What are the relative contributions of parental response to HBV and other reproductive factors to the sex ratio of offspring?

To address these questions, we used a weighted multiple linear regression analysis for estimating sex ratio (37). To simplify interpretation, we modeled an adaptation of the sex ratio statistic (number of males divided by the number of females) on a logarithmic scale so that positive values of the log of the sex ratio indicated more males and negative values indicated more females. We considered seven factors: HBsAg and anti-HBs in mothers and fathers, total number of pregnancies, fetal loss, and number of years of marriage (38). An initial analysis suggested that the presence of HBsAg in parents favors males and that anti-HBs in mothers favors females. However, anti-HBs in fathers, total number of pregnancies, number of years married, and fetal loss did not have important effects on sex ratio. We then decided to consider together the three HBV-related factors—HBsAg in fathers, HBsAg in mothers, and anti-HBs in mothers—as one "HBV response" variable. Anti-HBs in fathers was disregarded since its effect on sex ratio was negligible. This HBV response variable had three possible values: ‘‘−1” when both parents were HBsAg(−) and the mother was anti-HBs(−), ‘‘0” when both parents were HBsAg(−) and the mother was anti-HBs(−), and ‘‘+1” when either parent was HBsAg(+) [and anti-HBs(−)].
This combined variable is significantly related to sex ratio ($\chi^2 = 5.20$). In addition, fetal loss, although not significant as an individual factor, or in relation to anti-HBs(+) or HBsAg(-):anti-HBs(+) mothers, appeared to be important when considered in reference to HBsAg(+) mothers. The sex ratio of children born to HBsAg(+) mothers who had experienced abortions or stillbirths (or both) was noticeably lower than that of children of HBsAg(+) mothers who had not experienced fetal loss. Thus, a "combined HBsAg in mothers:fetal loss" variable took the values "−1" when the mother was HBsAg(+) with fetal loss, "0" when the mother was HBsAg(−), and "+1" when the mother was HBsAg(+) with no fetal loss. This combined variable was also important in predicting sex ratio ($\chi^2 = 2.61$). When only the two combined variables are considered, the analysis predicts sex ratios for five possible categories of parents. Table 2 shows these five categories with the total number of sons and daughters, the observed sex ratio, and the sex ratio predicted by a two-variable model with the following equation: predicted sex ratio = $\exp \left[ 0.319 + 0.296 \times \text{HBV response} + 0.553 \times \text{fetal loss} \right]$.

By observation, the observed and predicted sex ratios are nearly equal. However, the model is descriptive and requires further testing with another set of data (39). Fetal loss in HBsAg(+) mothers has only marginal statistical significance, based on a relatively small sample size, and is indicative of the tentative status of this model. Nonetheless, the results of this analysis reject the hypothesis that the relationship between parents’ responses to HBV and secondary sex ratio can be accounted for by other factors thought to affect sex ratio such as birth order, parental ages, and number of years of marriage. In addition, the analysis suggests some new hypotheses. (i) Anti-HBs has an effect on sex ratio only when it is present in mothers with HBsAg(−) husbands but not when it is present in fathers only. (ii) Fetal loss is important only in HBsAg(+) mothers and, when it occurs, is accompanied by a decrease in the generally high sex ratio of offspring of other HBsAg(+) couples. This suggests that, since HBsAg(+) mothers without fetal loss have a very high proportion of sons, those with fetal loss experience a preferential loss of males.

The regression analysis also suggested that the three original categories of HBV response be changed to the following: (i) either parent HBsAg(+):anti-HBs(−), (ii) both parents HBsAg(−):mother anti-HBs(−), and (iii) both parents HBsAg(−):mother anti-HBs(+). Figure 3 shows that these new category definitions yield clearer distinctions between the least-squares fits for the regressions of sons and daughters as compared with the number of years of marriage than did the original definitions (Fig. 1).

**Other HBV-Sex Interactions**

In considering possible explanations for the association between parents’ response to HBV infection and sex ratio of offspring, we examined two other instances in which HBV seems to interact with sex.

1) There is a sex difference in response to HBV infection. In most populations where the prevalence of chronic carriers is high, HBsAg is detected from 0.2 to 5 times more often in males than in females (33, 40). Similarly, both postnecrotic cirrhosis and primary hepatocellular carcinoma are more common in males (21, 41-43), and, although lupoid chronic [HBsAg(−)] hepatitis is more common in females, HBsAg(+) chronic hepatitis is more common in males (44-46). It has been argued that such differences are due mainly to an increased exposure of males to HBV infection (47). However, we have recently demonstrated that, among patients with end-stage renal disease treated with chronic hemodialysis, sex differences in HBsAg prevalence are due to a sex difference in response to HBV. We controlled the exposure variable by studying the responses to HBV infection, by sex, among 77 patients with renal disease, all...
of whom became infected with HBV while receiving treatment at a single hemodialysis clinic (49). Among these patients, males had a 68 percent chance of remaining HBsAg(+) persistently once infected with HBV, whereas females had only a 33 percent chance. Conversely, females had a 55 percent chance of developing anti-HBs once infected with the virus, whereas males had only a 30 percent chance. These differences in response were significant and were not related to sex differences in age, underlying renal disease, or incidence of infection.

2) We found that the response of renal graft recipients to HBV infection before transplantation and the sex of their kidney donors was associated with the duration of graft survival. We studied the survival of 87 grafts in 79 patients whose responses to HBV infection were assessed before transplantation (49). Kidneys from HLA nonidentical male donors which were transplanted into male or female patients who had anti-HBs prior to transplantation had very short survival. Eight of nine such grafts were rejected within 4 months of transplantation. Survival of grafts from male donors was significantly longer in both uninfected patients and chronic carriers of HBsAg (median survival for both groups was more than 22 months). Among the few patients who received grafts from female donors, there were no significant differences in graft survival between anti-HBs(+) uninfected, and HBsAg(+) recipients (50).

How Can HBV Interact with Sex?

These two observations, together with the results of the Plati sex ratio study, led us to the hypothesis that there is cross-reactivity between HBsAg and a male-associated antigen (51). If HBsAg cross-reacts with a male-associated antigen, males would be more likely to recognize HBsAg as "self" and therefore would remain HBsAg(+) persistently. Females, however, would be more likely to recognize HBsAg as "foreign" and produce anti-HBs. This would result in the observed predominance of males among chronic carriers of HBV in dialysis patients and other populations. In kidney transplant patients, tolerance to HBsAg in kidney graft recipients would result in tolerance (that is, longer survival) of male tissues, whereas anti-HBs in a recipient would react with male antigens on renal allografts, resulting in early rejection of grafts from male donors.

Similarly, we can speculate that tolerance to HBsAg [reflected in the maintenance of HBsAg(+):anti-HBs(-) status] in pregnant women would result in lack of sensitization against male tissues developing within them, and therefore good survival of male fetuses. Anti-HBs in women, however, could react with male antigens and perhaps hinder fertilization by sperm bearing a Y chromosome or increase the probability of spontaneous abortion of male fetuses. HBsAg(+) males would have HBsAg in their semen which, conceivably, could protect Y-bearing sperm from the effects of anti-HBs or other "anti-male" antibodies in their spouses' reproductive tracts (52). The effect of fetal loss in HBsAg(+) women could be explained by unhindered replication of HBV in male fetuses, and this could result in a greater loss of male than female embryos.

The cross-reactivity hypothesis cannot explain all the observations from the Greek study. If the effects of HBsAg and anti-HBs were the only factors modifying sex ratio, HBsAg(+) couples would have more sons than anti-HBs(+) couples (53). However, the observation that, even among parents married more than 15 years, HBsAg(+) couples had the same number of sons but fewer daughters per family as compared to anti-HBs(+) couples suggests that human behavior is affected by hepatitis B infection. In this Greek community most younger women preferred small families and practiced some form of birth control. Also, in some parts of Greece a preference for sons (as observed in many other societies) has been reported (6). It is possible that parents chose to limit family size only after bearing a desired number of sons. If this were so, couples where both parents were HBsAg(-) and the mother was anti-HBs(-) might give birth to several daughters before achieving the desired number of sons while HBsAg(+) couples would have the desired number of sons early in marriage and, as a result, restrict the number of subsequent births and therefore the total number of daughters born. The sex ratio of offspring of HBsAg(-) couples with anti-HBs(-) mothers would be unaffected by this preference since these couples express neither of the factors (HBsAg, anti-HBs) affecting sex ratio. If these explanations are valid, then in this community biological and social factors interact to influence the secondary sex ratio.

Conclusions

We have observed an association between the types of response to hepatitis B infection in parents and the sex ratio of their offspring. In either parent HBsAg was associated with a high sex ratio of live births, while anti-HBs(+) in mothers was associated with a low sex ratio. Fetal loss in HBsAg(+) mothers was also related to a lower sex ratio. Other variables thought to affect sex ratio, such as paternal and maternal ages (or number of years of marriage) and total number of pregnancies, were not significantly related when considered in the context of HBV response and fetal loss.

Evidence from studies of sex differences in response to infection with HBV, and survival of transplanted kidneys in recipients with different responses to HBV, as well as the study of sex ratio reported in this article led to the hypothesis that there is cross-reactivity between HBsAg and a male-associated antigen. This hypothesis requires both experimental and epidemiological testing.

Very few biological factors that affect sex ratio have been identified. Studies of the kind reported here should be conducted on other populations (54), and the effects of additional infectious agents and biological factors on sex ratio should be evaluated.

References and Notes

5. In many countries, including the United States, there is still an excess of males at reproductive age. The sex ratio in a population at this time depends on specific mortality figures.
7. Among the areas with populations reported to have sex ratios above or below the norm are Chile (103.0), Hong Kong (109.1), Aden (117.0), Korea (116.0), Gambia (116.2), Greece (113.0), and the Republic of South Africa (people of Asian origin only) (101.2)(1, 6).
16. The expression of the "sex ratio" condition also appears to depend on the genotypes of both the host and the infectious agent (16).
21. B. S. Blumberg, B. Leenheer, W. T. London, B.


27. This study was conducted by J. E. Hesser from our laboratory in collaboration with our Greek colleagues, I. Economouoti, S. Hadziyannis, and V. Sheard.

28. A similar association has been observed in French families [P. Cazal, J.-M. Lemaire, M. Rohner-Levy, Rev. Fr. Transfut. Immunohematol. 19, 377 (1976)].

29. Serums were tested for HBsAg by radioimmunoassay using a modification of the method of Ling and Overby, immuno diffusion, reactivity electrophoresis, and passive hemagglutination inhibition by the Hepanosticon (Organon) method. Anti-HBs was determined by passive hemagglutination. Sex ratios were compared by the two-by-two chi-squared test, numbers of sons and daughters by the Mann-Whitney U statistic, and linkage and linkage analysis by the analysis of covariance [C. M. Ling and L. R. Overby, J. Immunol. 169, 834 (1977)].

30. There was no difference between the couples reported by Hesser et al. (18, 19) and those analyzed in this study. We excluded the following: three couples in which the anti-HBs status of the HBsAg(+) parent was uncertain (five sons, one daughter), two couples in which both the father and mother were HBsAg(+) but the anti-HBs information was incomplete (13 sons, 4 daughters). We added the following: one couple in which the father was HBsAg(+) and the mother was not tested (two sons, two daughters), eight childless couples in which both parents were HBsAg(-) and one parent was anti-HBs(+), 13 parents were HBsAg(+) and two parents were HBsAg(-) and one parent was anti-HBs(+), 29 parents were HBsAg(-) and 22 parents were HBsAg(+), 8 parents were HBsAg(-), and 22 parents were HBsAg(+).

31. The HBsAg(+) category includes couples in which one parent was HBsAg(+) regardless of the test results of the other parent.

32. There are different antigenic subtypes of HBsAg, which may occur in one population but rarely in another. The presence of HBsAg and anti-HBs in one person probably represents antigen of one subtype and antibody to another.


34. We have made similar observations on an additional population from Mali, West Africa. The fathers of these families were not studied, however.

35. The data on parental ages and pregnancy and birth orders were available for ~ 90 percent of the 633 children reported. We monitored monthly for the presence of HBsAg and anti-HBs in their sera for long periods of time.


37. Most of these grafts were from cadaver donors, but the association of duration of graft survival with responses to HBV infection was independent of whether the donors were alive and related or whether the donors were cadavers. Although the early rejection of cadaver kidneys by anti-HBs(+) recipients has been observed by others, the specific association with grafts from male donors has not yet been confirmed.

38. All three studies involve populations in which the major HBsAg antigenic subtype is ay rather than ad. There is some indication from studies in the Solomon Islands (17) of a relation between the ad subtype and an alteration of secondary sex ratio in the opposite direction. In this way the genotypic composition of the host and the infectious agent would be important in determining the expression of this sex ratio effect, as seen in the transplantation.


40. The sex ratio is the case in the population from Mali, West Africa.

41. Preliminary results of sex ratio studies in African, Papua New Guinea, and Greenland are similar to those from the Piatni study; HBsAg is associated with higher sex ratios, while anti-HBs is associated with lower ones.

42. The sex ratio is the number of males per 100 females.


44. Supported by grants CA-06551, RR-05339, and CA-06927 and by an appropriation from the Commonwealth of Pennsylvania. We thank Dr. Ioanna Economouidi, director of the Hellenic Red Cross Blood Transfusion Center, Dr. Stephenos Hadziyannis, and the Hellenic Red Cross for their work in gathering and recording the Greek data, and the members of the Division of Clinical Research for their counsel and technical assistance.
SEX DIFFERENCES IN RESPONSE TO HEPATITIS B VIRUS

I. History

BARUCH S. BLUMBERG

Sex differences related to responses to hepatitis B infection are reviewed. In most human populations there is a higher prevalence of chronic carriers of hepatitis B virus (persistently HBsAg+) among males than females. Females are more likely than males to produce anti-HBs in response to infection. Diseases associated with increased frequencies of carriers are more prevalent among males. The response of parents to hepatitis B virus (HBV) infection appears to affect the sex ratio at birth of their offspring. Couples in which either parent is a carrier have higher sex ratios (higher proportion of males) compared with couples in which neither parent is HBsAg+. Couples in which the mother is anti-HBs+ have children with lower sex ratios than either carriers or uninfected couples.

Frequency of HBsAg carriers in males and females: Original observations. Soon after the discovery of Australia antigen (now known as hepatitis B surface antigen, HBsAg) in the serum of an Australian aborigine, we found that there were larger numbers of male than female carriers (1). In 1972 the then-known data on the distribution of the carrier state in non-hospitalized patients and in several groups of patients with diseases associated with Australia antigen were assembled (2). In all of these (with one exception) there was a higher proportion of male than female carriers and this difference was most striking in the younger age groups.

These results were consistent with the findings of Childs and his colleagues who had collected information from the records of Johns Hopkins Hospital from 1895 to 1955 on the sex of children admitted to the hospital with the diagnosis of bacterial meningitis or septicemia (3). They found that for nearly all of these conditions, there was a higher frequency in males than in females. This difference was most striking in the younger age groups. They further found that after the introduction of antibiotics for the treatment of these infections, the difference in mortality between males and females actually increased, although the absolute mortality for both sexes had decreased. They interpreted these data to mean that young females were more resistant to infection by a large number of species of bacteria, that once infected they responded to infection better than males, and when given appropriate therapy, they responded better to treatment.

Childs and colleagues then proposed a hypothesis based, in part, on the genetic concept of “Lyonization”; that is, for female cells there is a random inactivation of one X chromosome. They postulated the presence of genes controlling the production of antibodies against bacteria on the X chromosome. Because
of the random inactivation of X chromosomes, females would be more heterogeneous with respect to antibody formation and, therefore, have a greater variety of possible responses to infection than males. Males would have only a single kind of X chromosome and, consequently, less possibility of variation.

**Frequency of anti-HBs in males and females:**

Original observations. Subsequently, in collaboration with Alberto Vierucci and his colleagues, we studied an extensive collection of sera obtained from patients treated at the thalassemia center in Ferrara, Italy. Thalassemia patients produce high titers of anti-HBs, presumably as a consequence of their repeated immunization by HBsAg present in the blood used for transfusion. Using immunodiffusion, a relatively insensitive testing method, Vierucci et al found that, in general, females were more likely to develop anti-HBs antibodies and males were more likely to become carriers (4).

**Transmission of HBV from parents to children.**

Family studies were a major part of the early investigations of Australia antigen. In 1972 we found that in completed or partially completed families, there was a larger proportion of carriers among the offspring when mothers rather than fathers were carriers (5). This suggested that transmission from the mother to the offspring was a more likely event than transmission from the father to the offspring. Additional weight was given to this explanation when Mazzur et al reexamined families by using newly developed techniques for determining the subtypes of HBsAg (6). They found that in families in which either the mother or father was a carrier, the offspring nearly always had the same HBsAg subtypes as carrier mothers, but were often different from carrier fathers. This was consistent with the explanation that mothers but not fathers often transmitted hepatitis virus to their offspring. This study on completed families suggested that infection from the parents (primarily mothers) could occur when the children were quite young and that the children would remain carriers for a long time.

Direct evidence was obtained by Schweitzer (7) and subsequently others in their studies of mothers who had HBsAg positive (HbsAg+) hepatitis during the third trimester of pregnancy or at the time of delivery. They found that there was a much greater likelihood that the offspring of such mothers would become persistently HbsAg+ within weeks or months of birth than if the mother did not have hepatitis and HBsAg in her blood at the time of delivery. They did not find a high frequency of transmission by asymptomatic carrier mothers. Shortly afterwards, investigators in Asia found that women who were asymptomatic carriers during pregnancy were very likely to infect their infants, and such babies had a high risk of becoming carriers (8,9). This was particularly true if the mother had a high titer of HBsAg or was HBeAg+.

There have now been studies in several parts of the world, and these show that the frequency of maternal transmission varies from place to place (9-11). The time at which mother-to-child infection takes place could occur in utero, during birth, or in the early postnatal period. In Taiwan, intervention therapies at birth—delivery of the infant by cesarean section or administration of hepatitis B immune globulin (HBIg)—have not been effective in preventing maternal transmission (12), but prophylaxis with HBIg in Holland appears to have been effective (13).

These findings focused attention on early parent-child relations and led to systematic studies of biological events related to the infective status of parents. Before describing these, it may be useful to review some additional information on the sex relatedness of phenomena associated with hepatitis B.

**Observations on diseases associated with HBsAg.**

There are several diseases in which the frequency of HBsAg carriers is higher than in the general population or other diseases. These include chronic hepatitis (14), postnecrotic cirrhosis (15), primary hepatocellular carcinoma (PHC) (16), Down's syndrome (17), chronic renal disease treated with hemodialysis (18), some forms of leukemia (19), Hodgkin's disease (20), lepromatous leprosy (21), polyarteritis nodosa (22), and mixed cryoglobulinemia (23). For some of these, HBV is related to the etiology and/or pathogenesis; for others, abnormalities of the immune system are associated with an increased susceptibility to become a chronic carrier when exposed to the virus.

Each of these diseases, however, has a higher prevalence among males than females. Chronic active hepatitis associated with hepatitis B is more common in men, whereas chronic active hepatitis not associated with hepatitis B is more common in women. Postnecrotic cirrhosis is 3 to 4 times more prevalent among men than women, and the incidence of PHC is 4 to 9 times higher in men than women. The carrier rate is increased among patients with lepromatous leprosy, and males are much more likely than females to develop the lepromatous form. The other polar form of leprosy, tuberculoid leprosy, is not associated with persistent HBsAg and is equally prevalent in males and females. Persistent HBsAg is significantly more common in
Responses to Hepatitis B Virus

FIGURE 1. The probability of male or female patients who became infected with HBV in a single hemodialysis unit, remaining HBsAg- once they had converted from HBsAg-. Males with chronic renal disease have a 68% probability of becoming chronic carriers when first detected HBsAg-, whereas female patients have a 33% chance (28).

Down's syndrome patients than in other patients with mental retardation who reside in the same institution (24), and there is a male excess in Down's syndrome. A similar male excess is also found in the myelogenous leukemias, Hodgkin's disease, chronic renal disease leading to endstage kidney failure, mixed cryoglobulinemia (a disease associated with hepatitis B immune complexes), and periarteritis nodosa, many cases of which appear to be etiologically and pathogenetically related to hepatitis B infection.

It may also be pertinent that in one study HBsAg was localized by immunofluorescence in the testes (25). There is testicular localization of the primary disease process in several of the above mentioned diseases. Male Down's syndrome patients are generally sterile and have small atrophic testes, whereas females with Down's syndrome can bear children. The testes are said to be more commonly involved than ovaries in certain forms of leukemia. The testes are a site of concentration of Mycobacteria leprae (the causative agent of leprosy) in patients with the lepromatous form of leprosy, and male patients with lepromatous, but not tuberculoid, leprosy have testicular atrophy and reduced fertility. Female patients with lepromatous leprosy also have reduced fertility, but this appears to have a different cause (26).

Chronic renal disease and hemodialysis. For several years we have conducted a systematic study of the sera from patients with endstage renal disease who were undergoing chronic hemodialysis. Twice a month serum is collected from each patient and staff member at three commercial dialysis units in the Delaware Valley and tested for HBsAg, anti-HBs, and other factors related to hepatitis infection. From its inception, one of the renal dialysis units was designated to serve HBsAg- patients, and on this unit many of the patients either entered infected or became infected with HBV after treatment had begun. For example, patients without protective anti-HBs antibodies, who had been exposed on this unit for one year, had an 80% probability of becoming infected with HBV (27).

The accumulated sequential data on the patients in the renal dialysis unit provided a body of data for re-examining the differences between the sexes with respect to becoming carriers of HBsAg or developing anti-HBs. London and Drew (28) identified all patients in the unit who became infected with hepatitis B virus (as measured by the development of HBsAg in their blood). With a lifetable analysis, the probability of remaining a

![Figure 2. The probability of the same patients not developing anti-HBs once detected HBsAg+. About 55% of the female patients are likely to eventually develop anti-HBs compared with 30% of the males (28).](image-url)
carrier or of developing antibody was determined for each of the sexes separately. The results are shown in Figure 1. There was a significantly higher probability that males rather than females would remain carriers for an indefinite period. Similarly, there was a significantly higher probability that once infected, females would develop anti-HBs (Figure 2). The general conclusion from the study of this special group of patients is similar to the earlier findings in the thalassemia patients; that is, males are more likely to become carriers of HBsAg and females to develop antibody (anti-HBs) once they have been infected.

Mazzur had suggested that the higher frequency of HBV carriers in males could result from behavioral differences between males and females (29); that is, the life activities of males more often bring them into contact with hepatitis virus and, as a consequence, they are more likely to become infected and to become carriers. The design of the renal dialysis study minimizes this effect, since the behavior patterns of males and females on a renal dialysis unit are probably not greatly different from each other, and comparisons were made only between persons who became infected after initiation of dialysis treatment. Using a similar study design, Szmuness has confirmed these observations in other dialysis units (30).

II. Parental Responses to HBV Infection and the Secondary Sex Ratio of the Offspring

Early studies and generation of hypotheses. By 1970 it was apparent to us that hepatitis B infection, either persistent or acute, during pregnancy might affect the course of the pregnancy and/or the fetus. To that end, we studied over 800 pregnant women and 700 of their babies born at the Holy Family Hospital in New Delhi, India (31). By use of the immunodiffusion technique, 7 HBsAg+ pregnant women without clinical or laboratory signs of hepatitis were identified. The offspring of these women were all healthy and without congenital anomalies. Specifically, they did not differ from the children born to HBsAg− mothers with respect to length, weight, or head circumference. The HBsAg+ mothers, however, did differ significantly from HBsAg− mothers in two respects: they were younger and had longer gestation times. In addition, there was a suggestion of reduced fertility. None of the HBsAg+ women (by history) had been pregnant more than three times, whereas 20% of the HBsAg− controls had had three or more children. We suggested in the report of that study that the findings of younger age and fewer children among carrier women might represent either an effect of shorter time in the child-bearing age or relative infertility of older HBsAg+ women.

Earlier, in collaboration with Friedlaender, we had investigated the segregation of HBsAg in families living on Bougainville in the British Solomon Islands (32). Although the information for this study was not collected in a manner that would permit formal analysis of fertility or sex ratio of offspring, an exploratory study was done for the purpose of generating hypotheses. Differences in family size and sex ratio of offspring for certain matings led to the formulation of two hypotheses (33):

1. The fertility in matings in which one parent or the other has HBsAg will be different from one another, and from matings in which neither parent has HBsAg.
2. The sex ratio of the live births from matings in which one parent or the other has HBsAg will be different from one another, and from matings in which neither parent has HBsAg.

Studies in Plati, Greece. These hypotheses were tested in a field study carried out in 1973 by Hesser and her colleagues (34) in Plati, a village in Greek Macedonia. A significant increase in the secondary sex ratio (number of male/number of female live births × 100) was observed if either parent was HBsAg+ (sex ratio = 185) compared with the offspring of couples in which both parents were HBsAg− (sex ratio = 113). Significant differences in family size were not noted.

Later, after we had observed that patients who were anti-HBs+ prior to kidney transplantation had responses to allografts opposite from chronic carriers (to be discussed in greater detail in the following article), we reanalyzed the data from Plati. In the second analysis carried out by Drew et al (35), the families were divided into three groups:
Table 1. Number of male and female live births according to the responses to HBV of parents*

<table>
<thead>
<tr>
<th>Parent's response to HBV</th>
<th>Couples (No.)</th>
<th>Live births</th>
<th>Sex ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Either parent HBsAg + :</td>
<td>33</td>
<td>60 (1.8 ± 0.2)†</td>
<td>24 (0.7 ± 0.1)</td>
</tr>
<tr>
<td>anti-HBs –</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both parents HBsAg – :</td>
<td>29</td>
<td>51 (1.8 ± 0.2)</td>
<td>35 (1.2 ± 0.2)</td>
</tr>
<tr>
<td>anti-HBs –</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both parents HBsAg – :</td>
<td>154</td>
<td>24 (1.6 ± 0.1)</td>
<td>22 (1.4 ± 0.1)</td>
</tr>
<tr>
<td>either parent anti-HBs +</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data from reference 35.
† Numbers in parentheses indicate the mean number of live births per couple ± the standard error.
‡ Numbers in parentheses indicate 5% confidence limits of sex ratio.

1. Families in which either parent was HBsAg+:anti-HBs–
2. Families in which both parents were HBsAg–:anti-HBs–
3. Families in which both parents were HBsAg– and either parent was anti-HBs+

As shown in Table 1, this categorization demonstrated a very high sex ratio (250) in the offspring of HBsAg+ parents, an intermediate sex ratio (146) in the children of the HBsAg–, anti-HBs– parents, and the lowest sex ratio in the offspring of the anti-HBs+ parents (109). Further analysis showed that anti-HBs in mothers, but not in fathers, was associated with the lower sex ratio. Other variables thought to influence secondary sex ratio were examined by both Hesser et al (34) and Drew et al (35). Parental age, total number of pregnancies, birth order, child-to-parent transmission of HBV, and socioeconomic factors were evaluated and could not account for the observed sex ratio differences.

Similar studies have now been completed on the island of Karkar off the coast of New Guinea and in two communities in Greenland. In these populations secondary sex ratios are also associated with the HBV responses of their parents and the sex ratios are similar to those observed in Greece (unpublished data).

There may also be a fertility effect. In both Plati and Karkar, the number of sons born to each mating category (carrier parents or antibody positive mothers) was about the same (1.6–1.8 in Plati, 2.0–2.6 in Karkar), but the number of daughters per couple differed significantly (0.7 in HBsAg+ couples, 1.4 in anti-HBs+ couples in Plati, 2.3 in HBsAg+ and 3.2 in anti-HBs couples in Karkar). This could be explained by a combination of biological and behavioral factors affecting family size. We suggested that in Greece, where a preference for sons is known to exist, couples would continue to have children until they had the desired number of sons. Because of the effect on sex ratio of the parents’ HBV responses, HBsAg+ couples would achieve this end with fewer daughters, whereas anti-HBs+ couples would have to have more daughters and more children altogether. Alternatively, HBV transmitted from a carrier parent might replicate more rapidly and be more lethal to female than male embryos, resulting in the increased secondary sex ratios of HBV carrier couples. Mothers with anti-HBs would protect embryos from HBV infection, thus eliminating selection pressure on female fetuses.

Further discussion of a biological hypothesis which could explain the various sex differences associated with hepatitis B is given by London in the following article.

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Hepatitis B Virus and Sex Ratio on Kar Kar Island

By Jean Drew,1 W. Thomas London,1 Baruch S. Blumberg1 and Susan Serjeantson2

ABSTRACT

Hypotheses formulated from a previous study in Greece concerning the relationship between responses to hepatitis B virus (HBV) infection in parents and the secondary sex ratio of their offspring were tested in the population of Kar Kar Island, Papua New Guinea. The results were similar to those observed in the Greek village population. Sex ratios were highest (115) when either parent was a chronic carrier of HBV (HBsAg) and lowest (60) when both parents were HBsAg(−) and the mother had antibody to HBsAg (anti-HBs). Couples in which both parents were antigen and antibody negative had offspring with sex ratios intermediate (84) between the antigen positive and mother antibody positive couples. As in Greece, the sex ratio effects were due mainly to differences in the number of daughters, not sons, in each family; either parent antigen positive couples had the fewest (2.3) and mother anti-HBs(−), father HBsAg(−) couples had the most (3.9) daughters per couple. As a consequence, the latter couples tended to have larger total family sizes. Possible mechanisms to explain these observations are discussed.

Various epidemiological studies have demonstrated an interaction between responses to hepatitis B virus (HBV) in parents and the sex ratio at birth of their offspring (secondary sex ratio). An association between hepatitis infection and secondary sex ratio (no. of sons/no. of daughters × 100) has been observed previously in populations from Brigg, Great Britain (Robertson and Sheard, 1973), Languedoc, France (Cazal, Lemaire, and Robinet-Levy, 1976), Plati, Greece (Hesser, 1974; Hesser, Economidou, and Blumberg, 1975; Drew et al. 1978) and the Solomon Islands (Hesser, Blumberg, and Drew, 1976). In the Greek population, study of the HBV-sex ratio relation (Drew et al. 1978; Hesser, Economidou, and Blumberg, 1975) revealed the following: a) The sex ratio of live births from matings in which either the mother or father was positive for the surface antigen of HBV (HBsAg) was significantly higher than the sex ratio from matings in which neither parent had HBsAg; b) Antibody to HBsAg (anti-HBs) in parents was associated with a sex ratio alteration in the opposite direction from that observed with HBsAg; the children of matings in which either parent was antibody positive and

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neither was antigen positive had a lower sex ratio than the offspring of either antigen positive or antigen and antibody negative parents. Parental ages and birth order did not account for the observed differences in sex ratio.

Differences in family size associated with parental responses to HBV infection were also searched for. The average family size of antigen positive parents was smaller than that of antigen negative parents, but the difference was not statistically significant. Observations in the Greek population generated two additional hypotheses: a) Antibody is associated with a decrease in sex ratio when it is present in mothers (with HBsAg(−) husbands), but not when it is present in fathers; and b) Fetal loss in antigen positive mothers is associated with a decrease in sex ratio.

In this report we test, in an additional population, the various hypotheses tested in and generated by the sex ratio study in Plati, Greece. The subjects of this study were residents of Kar Kar Island, Papua New Guinea. This population is similar to the one from Plati in that it is a defined population in which hepatitis B infection is endemic. However, geographical, racial, socioeconomic, and cultural differences between the two groups are great. Thus, we expected that even if the association between HBV and secondary sex ratio was a consistent phenomenon, its expression might be different in the two populations.

**Materials and Methods**

The people of Kar Kar Island have been studied as part of the International Biological Programme (IBP) and the methods used have been described elsewhere (Walsh, 1974; Hornabrook, 1974). Prior to commencement of the IBP investigations, a study of the demography of the whole population of Kar Kar Island was undertaken (Hornabrook, 1974). During the demographic survey, live birth information was collected by the following procedure: "Field workers enumerated each dwelling on the island and recorded the inhabitants. Each individual was allotted an identification number, coded on the basis of village and sex. The marital state and offspring were recorded. The ages were noted separately depending on the source or basis on which estimations were made. The baptismal records of the Catholic mission, the hospital records of birth, and the ages as noted in the census or tax registers, were noted whenever available. The forms obtained by the field workers [were] checked and rechecked and coded for computer analysis" (Hornabrook, 1974). The Kaul village complex, a group of four villages in the northwestern part of
the island, was selected for more extensive study. In a census conducted by IBP personnel in 1969 the four villages had a total population of 1286. Every resident of the village complex received a medical examination, and a sample of blood was collected for genetic, biochemical and serological studies. On presentation for medical examination, subjects were asked once again the number of living and dead children. The present investigation includes subjects from the Kaul complex and four additional villages: Dorogodan, Langlang, Orora and Mangar. During the IBP studies, family relationships were not established and, in some cases, the total number of live births reported in the demographic and medical surveys were disparate, due to some inevitable loss of accuracy with the interview technique. Therefore, one of us (SS) revisited Kar Kar Island, before the tests of hepatitis B serology were done, to determine marital and pedigree relationships and to establish more accurately any previously unrecorded adoptions in the eight villages included in the present study. Although written records were not available to confirm all of the information obtained by interview, the comparisons made in this paper are within the population and should not be biased by the methods of data collection. Furthermore, polygamy is not practiced on Kar Kar and marriages are stable (Hornabrook, 1974).

![Fig. 1. Frequencies of HBsAg and anti-HBs by age and sex in the Kar Kar population.](image-url)

**Fig. 1.** Frequencies of HBsAg and anti-HBs by age and sex in the Kar Kar population.
Complete information on live births and serum samples from at least one parent were available for 239 couples. Of these, 146 couples were mother HBsAg(−):father untested and were excluded from analysis because of the probability that some of the untested fathers were HBsAg(+).

Serum samples were tested in our laboratory for HBsAg by radioimmunoassay (Ausria II (Abbott Laboratories, N. Chicago, IL)) (Ling and Overby, 1972) and for anti-HBs by passive hemagglutination (Vyas and Shulman, 1970; Juji and Yokochi, 1969). Evidence of exposure to HBV (HBsAg(+) and/or anti-HBs(+)) was found in 47% of the subjects tested (Figure 1). A higher proportion of males than females was HBsAg(+)).

Sex ratios were compared by the two-by-two Chi-squared test. The one-tailed test is appropriate for these studies, since the direction of the differences was predicted by the findings in Plati. The number of daughters per couple was compared by the Mann-Whitney "U" test.

### Results

The outcomes of the various types of matings with respect to the responses of patients to HBV are shown in Table 1. We had predicted on the basis of our earlier studies in Plati, Greece that the sex ratio of offspring of couples with either the mother or father antigen positive (HBsAg(+) would be higher than that of couples with both parents antigen negative (HBsAg(−)). The results fit the prediction (Table 1, row I vs. row II, 115 vs. 79; p = .051 (one-tailed)). We then tested a second hypothesis derived from the observation in Plati, that anti-HBs would be associated with a sex ratio alteration opposite from that associated with HBsAg. The results in Kar Kar supported the hypothesis (Table 1, row I vs. row II B, 115 vs. 77; p = 0.052 (one-tailed)). Furthermore, as in the Plati study, this observation was based on a difference in the mean number of daughters, not sons, per couple. Antigen positive couples had about the same mean number of sons (2.6 vs. 2.5; Table 1, row I, column A vs. row II B, column A) but significantly fewer daughters (2.3 vs. 3.2; Table 1, row I, column B vs. row II, column B) than antibody positive couples (p = 0.021, one-tailed).

Results of the analysis of the Plati data suggested that antibody is associated with a decrease in sex ratio when it is present in mothers (with antigen negative husbands) but not when it is present in fathers. In Kar Kar the findings were similar (Table 2). The lowest sex ratio and the most daughters per couple (3.9) were found in the offspring of matings in which both parents were antigen negative and the mother was antibody positive.
Male and Female Live Births According to Parents' Response to Hepatitis B Virus.
Data from Kar Kar Island, Papua New Guinea

### Table 2
Effects of anti-HBs in Either or Both Parents on Sex Ratio and Family Size

<table>
<thead>
<tr>
<th></th>
<th>Couples</th>
<th>Sons n/couple</th>
<th>Daughters n/couple</th>
<th>Children n/couple</th>
<th>Sex Ratio</th>
<th>Sex Ratio/Sex Ratio of Antigen and Antibody Negative Parents*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Either parent antigen positive (HBsAg(+)</td>
<td>24</td>
<td>2.6 ± .46</td>
<td>2.3 ± .41</td>
<td>4.9</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>II. Both parents antigen negative (HBsAg(−))</td>
<td>69</td>
<td>2.4 ± .22</td>
<td>3.0 ± .27</td>
<td>5.4</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>A. Both parents antibody negative (anti-HBs(−))</td>
<td>21</td>
<td>2.1 ± .41</td>
<td>2.4 ± .54</td>
<td>4.5</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>B. Either or both parents antibody positive (anti-HBs(+))</td>
<td>48</td>
<td>2.5 ± .26</td>
<td>3.2 ± .31</td>
<td>5.7</td>
<td>77</td>
<td></td>
</tr>
</tbody>
</table>

*The last column shows the sex ratios in relation to the sex ratio of offspring of uninfected (HBsAg(−), anti-HBs(−)) parents, which is assumed to be the sex ratio of the population without the influence of HBV infection.
Hepatitis B Virus and Sex Ratio

Jean Drew, W. Thomas London, Baruch S. Blumberg and Susan Serjeantson

Matings in which the father had anti-HBs resulted in fewer daughters per couple (2.9) and a sex ratio closer to that found in the offspring of antigen and antibody negative matings (Table 2, row III). Curiously, antibody in both parents was associated with equal numbers of male and female births.

It was possible that differences in parental ages could have accounted for the observed sex ratio differences, since the frequencies of HBsAg and anti-HBs vary with age (Figure 1). To address this matter, we examined sex ratio of live births of "completed" families only. We defined "completed" families as those in which the mother was at least 45 years old and presumably had terminated her reproductive activity. Table 3 suggests that, as we saw in Plati (Drew et al. 1978), the association of antigen with higher sex ratios and fewer daughters and that of antibody with lower sex ratios and more daughters is apparent even in this very small subgroup of children of older parents (p = 0.002; one-tailed).

Reliable information was not available on mothers' age at the time of the birth of each of their children or on birth order. By examining the age of the mothers at the time the data were collected and the number of sons and daughters born to each woman, it appeared that the older the woman the more sons she had, regardless of mating type. The number of daughters showed a similar increase with age of mothers among antigen negative couples, but not among antigen positive couples. To test the hypothesis that the number of daughters born to antigen positive couples increased at a different rate than those born to antigen negative or antibody positive couples, we estimated the linear least squares fits for the regressions of the numbers of sons or daughters vs. mothers' age for each of the three mating types. Analysis of covariance (not shown, available on request) revealed that the antigen positive couples had significantly fewer daughters than the antibody positive couples (see Table 1 for definitions of mating types). Also, antibody positive and antigen and antibody negative couples had a steadily increasing number of daughters with increasing maternal age, whereas antigen positive couples had a lesser increase in daughters. The number of sons among antigen positive couples increased with maternal age at about the same rate as among antigen negative couples.

Although the reliability of the number of miscarriages reported per woman is uncertain, we examined the data to see whether women who reported fetal loss had different sex ratios of offspring than those who did not. We did not observe significant differences within each mating type or for the entire population of women with reproductive information.
One of the purposes of the Plati study was to test the hypothesis that differences in fertility or family size are associated with the response of parents to HBV. In Plati the average family size was small (less than three children per couple), and significant differences were not observed. There was a suggestion of an increase in total number of live births among the anti-HBs(+) couples (3.0) compared with the HBsAg(+) couples (2.6) as a result of a decreased number of daughters and an equal number of sons born to the antigen positive parents. In Kar Kar, the family sizes are larger and the differences, which are in the same direction as those seen in Plati, are also larger (Table 1, column D). The maximum family size is observed when both parents are antigen negative, the father is antibody negative, and the mother is anti-HBs(+) (6.2 children/couple) (Table 2). This is significantly greater than the average of 4.9 children born to antigen positive couples. The regressions of the number of sons and daughters on maternal age for each mating type suggest that by age 45, each HBsAg(+) couple would have on the average 1.3 fewer daughters than either couples in which both parents are HBsAg(−), anti-HBs(−) or couples in which both parents are HBsAg(−) and either parent is anti-HBs(+). The actual data (Table 3) on completed families shows that HBsAg(+) couples had on the average 1.5 daughters compared with 3.5 for antigen and antibody negative couples and 4.5 daughters for antibody positive couples.

**Discussion**

The data presented here suggest that the relation of response to HBV in parents to sex ratio of offspring observed in Plati, Greece is not an isolated phenomenon. On Kar Kar Island, as in Plati, HBsAg in parents was associated with a high sex ratio of offspring while anti-HBs was associated with a significantly lower one. In Plati and Kar Kar, this difference is due primarily to a difference in the mean number of daughters rather than the mean number of sons per family. In neither Kar Kar nor Plati could these observations be accounted for by differences in parental ages.

We have suggested previously that the various observations of an interaction between HBV and sex could be caused by cross-reactivity between HBsAg and a male-associated antigen (Drew et al. 1978; Drew, London, Lustbader, and Blumberg, 1978). In antigen positive mothers, immunological tolerance to HBsAg (as reflected by the maintenance of HBsAg positivity) would be accompanied by tolerance to male embryonic
and fetal tissues; antigen positive fathers have HBsAg in their semen (Ogra, 1973) which could protect y-bearing sperm from anti-male factors in their wives’ reproductive tracts. The development of anti-HBs in women would be accompanied by lack of tolerance to sperm bearing the y chromosome and male embryonic and fetal tissues.

Cross-reactivity between HBsAg and a male-associated antigen cannot, by itself, explain a deficit of female births not accompanied by an increase in male births among HBsAg(+) couples. In regard to Plati, we suggested that birth control practices and parental sex preferences could account for this observation (Drew, London, Lustbader, and Blumberg, 1978). If parents limited family size after having some desired number of sons, then antigen positive couples, having had a greater proportion of sons born among their first two or three children, would attempt not to have additional children. Anti-HBs(+) couples, on the other hand, having had more girls than boys among their first few children, would continue to have more children until they had had the desired number of sons. In Kar Kar, although there is some knowledge about modern forms of birth control, these are not regularly practiced (Stanhope and Hornabrook, 1974). There are many ways of limiting family size that could be in use including regulation of duration of lactation, coitus interruptus, anal intercourse, insertion of sponges or other barriers in the vagina, etc. Hence, based on our observations on hepatitis B virus and sex ratio, the hypothesis could be made that in Kar Kar there is a preference for male children and traditional birth control practices are used to achieve this aim. In this example a biological observation has been used to generate a hypothesis which can be tested using the methods of social anthropology.

Other explanations should also be considered. In 1977 we reported that among patients treated with chronic hemodialysis and infected with HBV, males were more likely to develop chronic HBs antigenemia while females were more likely to develop anti-HBs (London and Drew, 1977; London et al. 1977). Data from the same population showed that patients who developed anti-HBs experienced more severe but shorter-lived episodes of liver damage (alanine aminotransferase was elevated to higher levels but for shorter periods of time) than those who developed persistent antigenemia (London et al. 1977). There is evidence that most of the damage to hepatocytes in acute hepatitis B is produced by the immune response to infected cells rather than direct cell lysis by viral replication (Blumberg, Sutnick, and London, 1970). The severity and duration of liver damage and the types of host response to HBV could be related to differences in the rate of HBV replication. That is, rapid replication of
HBV in a cell would lead to release of many virus particles, stimulus of the immune system, destruction of HBV infected cells by T-lymphocytes and production of anti-HBs. Slow replication would result in prolonged release of small numbers of viral particles, gradual accumulation of particles in the circulation, little or no stimulus of the immune system to produce anti-HBs and, hence, persistent HBs antigenemia with little or no liver damage. The fact that females are more likely than males to develop anti-HBs (London and Drew, 1977; Szmuness, Harley, Ikram, and Stevens, 1978) therefore suggests that, in general, HBV replication may be faster in female than in male cells.

There is no direct information indicating that human embryos can be infected with HBV in utero. There is circumstantial evidence, however. High titer anti-HBs given at birth to babies born to mothers carrying infectious hepatitis B virions alters the response of these infants to infection but does not prevent infection (Stevens et al. 1981). Since hyperimmune globulin is effective in preventing infection if given at the time of exposure to HBV (Grady et al. 1976), the failure to prevent transmission to newborns suggests that infection may have occurred in utero. Secondly, a virus closely related to human HBV occurs in Pekin ducks (Mason et al. 1980). This virus, called duck hepatitis B virus (DHBV), is definitely transmitted vertically. It produces infection under commercial breeding conditions in duck embryos as early as five days after laying (O'Connell and London, unpublished data). If human early embryonic cells can be infected with HBV and if such infected cells die or are unable to differentiate normally, then in utero infection would lead to more rapid replication and spread of HBV in female than male embryos. This would result in reduced viability of female embryos and a decrease in the number of female offspring born to HBsAg(+) parents. Anti-HBs in a pregnant woman's serum and in the secretions of her reproductive tract could protect her fetus from being infected by HBV, or related agents, in the semen of an HBsAg(+) mate. If female embryos were at greater risk of a lethal HBV infection, protection by anti-HBs would result in a greater increase in the viability of female than male fetuses. This speculative hypothesis warrants experimental and epidemiological testing.

Biological factors responsible for sex ratio variations in human populations have generally been obscure. The present study describes a second population in which responses to HBV infection in parents have been significantly related to sex ratio of offspring. In addition, in this population there is a significant and large effect on fertility (carriers of hepatitis B virus have fewer children than parents with antibody). Further study of
the effects of HBV and other infectious agents on sex ratio and fertility may uncover useful clues to factors which influence these fundamental biological parameters.

ACKNOWLEDGMENTS

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LITERATURE CITED


Sex-Related Differences in Transmission of Hepatitis B Infection in a Melanesian Population

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KEY WORDS Hepatitis B virus, HBeAg, HBsAg

ABSTRACT Factors involved in the spread of hepatitis B virus (HBV) in the largely Melanesian population (N = 909) of Graciosa Bay, Ndeni, are examined. Based upon cultural information from Ndeni and ways in which HBV is spread in other populations, certain practices and interactions, predicted as effective routes of HBV infection, are analyzed. Most significant are father-to-son transmission and older-brother-to-younger-sibling transmission, indicating that males are instrumental in the transmission of HBV on Ndeni. Other possible routes of HBV transmission that are not shown to be significant on Ndeni are significant modes of disease spread in other cultures. Cross-cultural differences are discussed with regard to behavior, age of infection, and persistence of HBeAg (an antigen linked to HBV infection). Particularly with regard to maternal transmission, it is clear that the average age of infection combined with the degree of HBeAg persistence limits the number of infective mothers in this population. Cultural practices, however, may explain the disproportion of male carriers over 30 implicating a largely extinct set of culturally sanctioned practices involving very early exposure to HBV in boys and their age-related inability to make antibody to the virus.

This paper examines the spread of hepatitis B virus (HBV) in a largely Melanesian population living along the shores of Graciosa Bay. Graciosa Bay is located on the South Pacific island of Ndeni in the Solomon Islands (formerly Santa Cruz of the British Solomon Islands Protectorate). The data are based on census material and sera collected during a series of field trips conducted from 1973 through 1977 by the late Scott Mazzur.1 The study uses cultural information and knowledge about the possible routes of HBV transmission to predict routes of infection within families and other subgroups of the population. These predictions are then tested using serological and census-related data, and the apparent routes of HBV infection are determined. As the spread of infection is dependent upon certain behaviors, the results of this analysis also generate new hypotheses about behavior.

Hepatitis B is caused by a relatively small virus (42 nm) containing a distinctive and partially double-stranded circular DNA, an associated polymerase, and several distinguishable protein components. The DNA polymerase incorporates nucleotides into the incomplete single-strand portion prior to viral replication. Both the DNA and its polymerase are contained within a protein core known as the core antigen or HBeAg [Hoofnagle et al., 1974]. The outer surface of the virion is comprised of a second protein termed the surface antigen HBsAg, or initially, Australia antigen [Blumberg et al., 1967]. A third antigen, HBeAg, is typically found in conjunction with the DNA and its polymerase [Magnius et al., 1975] and is probably a component of the core.

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Scott Mazzur died in 1979. In accordance with her wishes, the Division of Clinical Research of the Institute for Cancer Research maintains the data base and records of her expeditions to Graciosa Bay.
Specific antibodies produced by the host are often present in the serum after infection with HBV. These antibodies are associated with the major proteins of the virus, HBsAg, HBeAg, and HBcAg. Anti-HBs, the antibody to HBsAg, protects the host against subsequent HBV infection [Blumberg, 1977].

There are a variety of responses to HBV infection:

1) Acute hepatitis followed by complete recovery and serologically marked by the appearance of HBsAg and anti-HBc initially, and succeeded by the appearance of anti-HBs and the disappearance of the two earlier markers of infection.

2) Acute hepatitis with 5–10% of cases developing the chronic carrier state and symptoms. Serologically, anti-HBs and anti-HBc persist.

3) Chronic hepatitis with symptoms of chronic liver disease. HBsAg and anti-HBs persist.

4) Asymptomatic carrier. Initially there are no symptoms. Serologically, HBsAg and anti-HBc persist. HBeAg may also be present and this appears to be well correlated with viral production and the ability of one human to infect another [Magnius et al., 1975]. These individuals are at increased risk of developing chronic liver disease and primary hepatocellular carcinoma.

5) Asymptomatic sustained antibody production. Serologically, no trace of HBsAg but persistence of anti-HBs [Blumberg, 1977].

Factors that help determine which response a given host will exhibit are probably both genetically related and dependent upon the age, sex, and health of the host [Blumberg and Hesser, 1975].

The probability of being infected with HBV is an equally important issue. Infection with HBV is due to direct contact with either an infected individual’s blood or secretions. Possible infection routes include injections with unsanitary needles, tattooing, sexual intercourse [Szmuness et al., 1975], sharing of chewed material [Leichtner et al., 1981], birth [Papaevangelou et al., 1974], and direct contact with menstrual blood [Mazzur, 1973].

The Graciosa Bay Villagers of Ndeni share a high risk for both HBV infection and chronic carrier status with the other inhabitants of the Solomon Islands [Blumberg et al., 1974]. The Graciosa Bay Villagers, though, are primarily Melanesian with a small amount of Polynesian admixture. They live in 14 continuous villages consisting primarily of leaf hut dwellings. Though several dialects and some distinct language differences exist, the villages are culturally homogeneous, and intermarriage between villages frequently occurs. Villagers are generally in good health despite the prevalence of malaria, respiratory illnesses, and limited native knowledge of infection.

Certain behaviors that could facilitate the spread of HBV are prevalent in the interactions of individuals on Ndeni based on age, sex, and family position. All children are breast-fed and, generally, mothers feed only their own children who are under 2 years of age. Between the ages of 2 and 5 years, children are weaned with premasticated food. While mothers are primarily involved in weaning children, other members of the family often participate. In particular, fathers, proud of young sons, are generally seen carrying their sons around the village.

Hepatitis B virus spread facilitated by homosexual intercourse is also a factor on Ndeni. Homosexual relations, as a form of sexual gratification, are culturally sanctioned, and sexual relations among both men and adolescent boys are prevalent [Davenport, 1964].

In the past, older men often engaged in intercourse with very young boys. Then, the men’s house was the foremost hut in the village. It was the place where men slept and adolescent boys ventured to have their first heterosexual encounters with the village concubines. Premarital sexual intercourse was strictly forbidden except in the men’s house, where both married and marriageable village women were banned. Almost complete public segregation of the sexes was also practiced and adultery was uncommon. As a result, homosexual relations, while not preferred over heterosexual encounters, were a major source of sexual gratification in adolescents and widowers [Davenport, 1964].

In the last 30 years, western missionary moralizing and government regulation has destroyed the village concubine system and reduced the men’s house to a vestige of its former role. Today, men’s houses are make-shift dwellings where adolescent boys and unmarried young men sleep and engage in sexual relations. Ironically, the missionary-inspired breakdown of the men’s house has been accompanied by the loosening of premarital sexual segregation so that today adolescent heterosexual promiscuity is commonplace [Davenport, 1965].
In addition to homosexual experiences, husbands engage in sexual intercourse with wives, and heterosexual contact may spread infection as it does in other societies [Szmusness et al., 1975]. Also, in keeping with tradition, boys spend much of their time with their fathers or with age peer groups. Here the sharing and chewing of betel nut between men and their sons, as well as the contact between young boys in play, may facilitate the spread of HBV. Girls, on the other hand, spend much of their time working in the fields with their mothers and little time chewing betel nut or playing with a group.

The high prevalence of HBV infection on Ndeni, the homogeneity of the villages, and the knowledge of virus transmission behaviors (i.e., the mode of weaning, betel nut chewing, sexual practices, and sex differences in child rearing) allowed us to make the following hypotheses prior to the analysis of HBV transmission on Ndeni: 1. Carrier (HBsAg(+)) mothers have significantly more infected offspring aged 0 to 2 years than noncarrier mothers. 2. Infants of anti-HBs(+) mothers show an antibody response due to acquired immunity. 3. Carrier mothers and/or carrier fathers have significantly more infected children aged 3 to 5 years than noncarrier mothers and/or fathers. 4. Older carrier children have significantly more infected sibs aged 0 to 2 years and 3 to 5 years than older noncarrier children have. 5. Due to sexual segregation practices, there is a stronger relationship between a father's carrier status and infection in his sons than between a father's status and infection in his daughters. 6. Men's house cohabitants are at greater risk of infection and carrier status than siblings or cohabitants who live at home. 7. Carrier husbands have significantly more infected wives than do noncarrier husbands.

MATERIALS AND METHODS

From 1973 through 1977 a census of the Bay Villages was compiled and updated by Scott Mazzur. All individuals who were serologically tested were listed in the census and all parents, spouses, and sibs were recorded for use in intrafamily infectivity studies. In addition, the residence of each individual was noted so that cohabiting relationships could also be studied with regard to HBV. One of us, William Davenport, completed a census of this same island population in the 1950s and this earlier census was used in both verifying and enriching the information in the later census. Subsequent visits to Ndeni by Davenport have enabled an assessment of the changes in the traditional life style so that an accurate and current description of village life on Graciosa Bay was obtained.

In 1974, 771 individuals were tested for HBsAg and anti-HBs and the frequency of these viral markers was reported [Mazzur and Jones, 1977a]. In 1977 a more comprehensive sera collection was undertaken, and 909 individuals, roughly 84% of the population, were sampled. In addition to HBsAg and anti-HBs, anti-HBc, HBeAg and anti-HBe were tested.

The serological analyses in this paper rely exclusively upon the 1977 sample with but one exception. In the analysis of acquired immunity, some mothers were only tested in 1974. As a result, the 1974 anti-HBs status of these mothers is analyzed and the results are discussed separately.

During both blood collections, vacutainer samples were obtained from adults while capillary tubes were used to obtain samples from infants and young children. HBsAg testing by a commercial reverse passive hemagglutination assay (RPHA) system (Auscell, Abbott) and anti-HBs testing by use of lyophilized HBsAg-coated red blood cells (Abbott) were done in the field. All initial results were confirmed in the field by repeating the test after neutralizing the sample with the appropriate antigen or antibody.

In 1977, samples of sufficient volume were sent to Dr. N. Nath, American Red Cross, Bethesda, Maryland for tests for anti-HBc activity by radioimmunoassay (CORAB, Abbott). Field identified HBsAg positive samples were retested by radioimmunoassay (Auscella, Abbott). Those with sufficient volume were tested for HBeAg and anti-HBe by rheophoresis (Austect, Abbott) and/or by radioimmunoassay by Dr. H.A. Field, Center for Disease Control, Phoenix, Arizona. Field identified anti-HBs samples were retested by hemagglutination (Electronucleons).

To describe this serological data, our report uses the terms HBsAg status and HBV outcome. HBsAg status describes an individual as a carrier (HBsAg(+)) or a noncarrier (HBsAg(-)). The HBV outcome describes...
three types of responses to contact with a hepatitis B infectious carrier: 1) not infected; 2) infected but not a carrier; and 3) carrier. Those who are not infected tested negative for all the antigen and antibody responses specific for HBV that are described above. Those who are carriers tested positive for the surface antigen. Those who are described as infected but not carriers tested positive for some markers of HBV infection but not HBsAg or HBeAg. Those individuals had produced anti-HBs and/or anti-HBc when infected with HBV.

In analyzing the serological data within the context of the census material all of those sampled are included. As a result, families with more members appear more frequently in the tables than families with few members. For example, in analyzing the association between a mother's HBsAg status and a 0- to 5-year-old child's outcome, the outcome of each child within the given age group is matched to the mother's HBsAg status. In this way, the status of women who have two children in this age group is listed twice, while the status of women with one child in this group is listed only once. As exposure and response to HBV among sibs vary, this approach is comprehensive. Furthermore, as larger families contribute more individuals to the population, it seems appropriate to score each individual rather than each family.

RESULTS AND DISCUSSION

Maternal transmission

Mothers' and/or fathers' HBsAg status was not associated with the HBV outcome of their children aged 0 to 2 years (see Table 1). Of 53 children ages 0 to 2, 48 were uninfected. Of the five children who were infected, four were HBsAg(−) and only one child was anti-HBs(−). All five of these infected children were the offspring of HBsAg(−) mothers, but two of these children had HBsAg(+) fathers.

The lack of detectable maternal transmission from mothers to children less than 2 years of age suggested that on Ndeni HBV was not spread from mother to child during pregnancy and birth or during the many months of breast feeding and related child care following birth. These findings are consistent with Mazur and Jones' [1977b] review of the 1974 data from Ndeni that compared the HBsAg status of all mothers with their children regardless of age. Maternal transmission, however, is an effective route of HBV infection in Africa [Maupas et al., 1981], Asia [Stevens et al., 1975; Okada et al., 1975] and other parts of the South Pacific [Dickie et al., 1982].

In places where maternal transmission occurs, perinatal infection is one way HBV is spread. In Taiwan, Stevens et al. [1975] reported an extremely high rate of maternal transmission. Of 158 babies born to asymptomatic HBsAg(+) women, nearly 40% (63 of 158) tested HBsAg(+) by 6–12 months of follow-up. To test a specific route of maternal transmission, Beasley and Stevens [1975] compared breast fed and bottle fed babies of HBsAg(+) mothers in Taipei and found no apparent evidence for transmission via breast feeding. Thus, Stevens et al. [1975] suggested that transmission probably occurred during labor and delivery.

A specific delivery practice does not seem to preclude maternal transmission. In the United States and in Europe transmission from asymptomatic HBsAg(+) mothers to their children does not occur even though delivery practices are similar to those in Taiwan. On Ndeni, where hospital care is not available, maternal transmission could not account for any infection among children under 2 years of age.

It is evident that HBsAg(+) women differ in the ability to transmit HBV to their infants. Schweitzer et al. [1973], in a 3-month follow-up of 21 children born to HBsAg(+) asymptomatic mothers, found only one HBsAg(+) child. Similar results were reported in studies from Europe (Skinhøj et al. in Denmark [1972] and Papaevangelou in Greece [1973], Thailand (Punyagupta et al. [1973], and the South Pacific (this study). Yet in Japan [Okada et al., 1975], the rate of vertical transmission from asymptomatic mothers to infants is similar to that reported by Stevens et al. [1975] for Taiwan. Similarly, in the United States Schweitzer et al. [1972] reported relatively high levels of transmission when mothers tested HBsAg(+) with acute hepatitis. In their study, seven of 26 women had HBsAg(+) children within 3 months of delivery.

HBeAg and maternal transmission

HBsAg alone is not enough of a predictor of maternal vertical transmission. Other factors involving the response of the host to
Transmission of Hepatitis B

TABLE 1. HBsAg status of parents and HBV outcome in children aged 0-2 years and 3-5 years

<table>
<thead>
<tr>
<th>HBsAg Father</th>
<th>Age of children</th>
<th>Outcomes in children</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carrier</td>
<td>Infected, not carrier</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>0-2 yrs</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>3-5 yrs</td>
<td>2</td>
<td>38</td>
</tr>
<tr>
<td>Negative</td>
<td>0-2 yrs</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3-5 yrs</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Positive</td>
<td>0-2 yrs</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3-5 yrs</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>Positive</td>
<td>0-2 yrs</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3-5 yrs</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: Anti-HBs(+) and/or anti-HBc(+) 

TABLE 2. Prevalence of HBeAg and anti-HBe in Graciosa Bay HBsAg(+) carriers

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>HBeAg(+)</th>
<th>Anti-HBe(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#</td>
<td>%</td>
<td>#</td>
</tr>
<tr>
<td>Unmarried females</td>
<td>11</td>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td>Married females</td>
<td>17</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>All females</td>
<td>28</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Unmarried males</td>
<td>24</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>Married males</td>
<td>24</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>All males</td>
<td>48</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>11</td>
<td>14</td>
</tr>
</tbody>
</table>

HBV infection, particularly during pregnancy and delivery, need to be considered. An important factor linked to HBV infectivity is HBeAg. Presence of HBeAg and absence of anti-HBe has been shown to facilitate horizontal HBV transmission [Shikata et al., 1977].

HBeAg was detected in 11 (14%) of 76 HBsAg(+) individuals tested. The level of anti-HBe was similar (16%). The distribution of HBeAg and anti-HBe was similar for both the 48 HBsAg(+) males and the 28 HBsAg(-) females tested (see Table 2).

Among the 17 married HBsAg(+) women who were tested, only one was HBeAg(+) while four (24%) were anti-HBe(+) (see Table 2).

The small percentage of HBeAg(+) mothers among the relatively small percentage (9-14%) of HBsAg(+) women of childbearing age (see Table 3) limits widespread transmission of HBV in our study population. Only two of the 53 children under 2 years of age had HBsAg(+) mothers (see Table 1).

In contrast, HBeAg is found in asymptomatic carriers at relatively high frequencies in Taiwan where maternal transmission is documented. In one study 20 of 62 carrier Chinese mothers (32%) were HBeAg(+) while only 2% were anti-HBe(+) [Beasley et al., 1977]. In this same study, 13 of the 42 HBeAg(-) mothers (31%) had HBsAg(+) offspring, while 17 of the 20 HBeAg(+) mothers (85%) had carrier children (see Tables 4, 5). In Europe, where vertical transmission of HBsAg is low, HBeAg is found in virtually none of the asymptomatic carriers tested, while anti-HBeAg is found at levels well over 50% [Eleftheriou et al., 1975; Magnus et al., 1975] (see Table 4).

Persistence of HBeAg through childbearing years

Among HBsAg(+) village children, however, the proportion of HBeAg(+) carriers is much higher than among older individuals of marriageable age. A comparison of married carriers (generally all those over 20) with
TABLE 3. Hepatitis B virus marker distribution by age and sex in the 1977 sample of the Graciosa Bay Village population

<table>
<thead>
<tr>
<th>Age of group</th>
<th>Sex</th>
<th>Carrier HBsAg (+)</th>
<th>Not infected</th>
<th>Infected, not carrier</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number and (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-2 yrs</td>
<td>M</td>
<td>1 (3)</td>
<td>33 (97)</td>
<td>0 (0)</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0 (0)</td>
<td>14 (93)</td>
<td>1 (7)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>u</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>3-5 yrs</td>
<td>M</td>
<td>5 (14)</td>
<td>24 (69)</td>
<td>6 (17)</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>2 (8)</td>
<td>21 (81)</td>
<td>3 (11)</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>u</td>
<td>0</td>
<td>13</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>6-10 yrs</td>
<td>M</td>
<td>12 (17)</td>
<td>25 (35)</td>
<td>35 (48)</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>2 (3)</td>
<td>39 (61)</td>
<td>23 (36)</td>
<td>64</td>
</tr>
<tr>
<td>11-15 yrs</td>
<td>M</td>
<td>6 (8)</td>
<td>23 (30)</td>
<td>47 (62)</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4 (9)</td>
<td>19 (43)</td>
<td>21 (48)</td>
<td>44</td>
</tr>
<tr>
<td>16-20 yrs</td>
<td>M</td>
<td>2 (8)</td>
<td>7 (27)</td>
<td>17 (65)</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>3 (14)</td>
<td>6 (27)</td>
<td>13 (59)</td>
<td>22</td>
</tr>
<tr>
<td>21-30 yrs</td>
<td>M</td>
<td>5 (11)</td>
<td>7 (16)</td>
<td>32 (73)</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6 (10)</td>
<td>19 (30)</td>
<td>38 (60)</td>
<td>63</td>
</tr>
<tr>
<td>31-40 yrs</td>
<td>M</td>
<td>10 (25)</td>
<td>5 (12)</td>
<td>26 (63)</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>7 (13)</td>
<td>9 (16)</td>
<td>39 (71)</td>
<td>55</td>
</tr>
<tr>
<td>41-50 yrs</td>
<td>M</td>
<td>7 (22)</td>
<td>3 (10)</td>
<td>21 (68)</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4 (16)</td>
<td>5 (19)</td>
<td>17 (65)</td>
<td>26</td>
</tr>
<tr>
<td>&gt; 50 yrs</td>
<td>M</td>
<td>5 (24)</td>
<td>0 (0)</td>
<td>16 (76)</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0 (0)</td>
<td>2 (13)</td>
<td>14 (87)</td>
<td>16</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>84 (11)</td>
<td>275 (38)</td>
<td>371 (51)</td>
<td>730</td>
</tr>
</tbody>
</table>

*Anti-HBc (+) and/or anti-HBd -f-.

unmarried carriers (generally those under 20) showed a four- to five-fold decrease in the proportion of HBeAg(+) individuals in the older, married HBsAg(+) carrier group (Table 2). In studying Japanese HBsAg carriers, Ohbayashi et al. [1976] also noted a decrease in the frequency of HBeAg with age (Table 4). However, Ohbayashi et al. [1976] reported frequencies of HBeAg for both adults and children that were much higher than those found on Ndeni. Among the HBsAg(+) Japanese studied, the percentage of HBeAg(+)) individuals under 20 was well over 40% (see Table 4). In comparison, the percentage of those HBeAg(+), among unmarried carriers on Ndeni, was well under 30% (see Table 2). Similarly, while the proportion of HBeAg(+) Japanese carriers does decrease from 41% at ages 11 to 20 to 17% at ages 21 to 40, the proportion of infectious HBsAg(+) Japanese adults remains at a level that is three times as great as the frequency of infectious HBsAg(+) adults on Ndeni (see Tables 2 and 4). In another Asian population, a Korean population with chronic active liver disease, Kim et al., [in preparation] reported even higher levels of HBeAg persistence (> than 20%) among HBsAg(+) adults (see Table 4). The persistence of HBeAg at levels greater than 15% among populations of Asian adult carriers [Beasley et al., 1977; Okada et al., 1976; Ohbayashi et al., 1976; Kim et al., in preparation] helps maintain a large number of infectious adult carriers in these populations (Table 4). The level of HBeAg persistence found in Asia, but not on Ndeni, is probably responsible for the significant amount of maternal, neonatal, HBV transmission reported in Asia but not apparent on Ndeni. In turn, population differences in the persistence of HBeAg may be directly related to factors such as the age of onset of infection.

Age at onset of infection related to outcome

At a time when mother and child contact is so intimate, it is fortunate that there are so few HBeAg(+) mothers in the Graciosa
Bay Villages because, once infected, children aged 0 to 2 are more likely to become carriers than older children. A significant difference in response (P = .04) was noticed when viral markers were compared in older children ages 3 to 5. A decrease in HBsAg(+) responses to infection was apparent: four of five infected 0–2-year-olds were HBsAg(+) (80%) while seven of 18 infected 3–5-year-olds (39%) were HBsAg(+), and only 14 of 72 infected 6–10-year-olds (19%) were carriers (Table 3). Very few of the children aged 0 to 2 were tested for anti-HBc because the serum samples were generally of insufficient volume. Comparisons between the 0–2-year-olds and the 3–5-year-olds using only HBsAg and anti-HBs data revealed the same significant difference in outcome between age groups as reported above.

This same pattern is seen in Senegal, where 34% of children under 2 years of age were infected with HBV and half of these were...
HBsAg(+) but by age 13, the prevalence of HBsAg among those infected is greatly reduced, and while 91% are infected only 13% of these are HBsAg(+) [Maupas et al., 1981]. Studies done in America in institutional settings have also shown a relationship between early exposure to HBV and increased likelihood of becoming a chronic carrier [Szmuness et al., 1970]. Similar results from Malo Island concerning newborns led Dickie et al. [1982] to conclude that perinatal exposure to HBV is likely to result in a failure to produce antibody and a lifelong HBsAg carrier status.

**Primary hepatocellular carcinoma on Ndeni**

Chronic HBV infection has been associated with liver disease including post hepatitic cirrhosis and primary hepatocellular carcinoma (PHC). There is also evidence that the onset, and possibly the pathogenesis, of HBV-related chronic liver disease may depend in part on the age at which chronic infection begins [Blumberg et al., 1975].

A review of medical records at a hospital on Kirra Kira, a nearby island, revealed the death of a 5-year-old village girl from primary hepatocellular carcinoma. The clustering of HBsAg(+) carriers within her nuclear family makes early exposure to HBV infection a likely component in this child's terminal illness. In 1977, a year or more after the girl’s death, her mother tested HBsAg(-), HBeAg(-), and anti-HBe(—). The lack of detectable HBeAg in the mother in 1977 may not be a good indicator of the mother's infectivity 5 to 10 years earlier when the girl and three surviving siblings were born. Two of the three surviving siblings are HBsAg(+) but were not tested for HBeAg and anti-HBe. The other sibling was HBsAg(—), anti-HBe(—), and anti-HBc(—) at age 7. The presence of only anti-HBc indicates exposure to HBV years before; and in a child of 7, this means early exposure to the virus. The carrier status of the two other young sibs are likely outcomes to early HBV infection. The father was HBsAg(—), anti-HBs(—), and anti-HBc(—). The absence of HBsAg in the father and its presence in the mother coupled with the markers exhibited by the children indicate maternal transmission as the likely pathway for infection in this family. It is conceivable that perinatal transmission of HBV resulted in immunologic tolerance in three of the four children, and that in one of these three young HBeAg(+) carriers, early HBV infection promoted the development of primary hepatocellular carcinoma. It is likely that as better medical services become offered on Ndeni, more individuals will be identified with PHC and other HBV-related liver diseases.

**Lack of acquired maternal antibody**

Six of the 30 children less than 1 year old had mothers who were anti-HBs(+) in 1977. Though four of these six children were only 6 months old, none showed an antibody response. A 7-month-old child and another under 1 year also showed no trace of acquired immunity. Two more children aged 8 months and just under 1 year had mothers with detectable anti-HBs in 1974. Neither of these children had a measurable anti-HBs response.

The lack of detectable acquired maternal antibody in children 6 months old or greater is consistent with reports from Senegal [Maupas et al., 1981]. While 30% of newborn Senegalese babies are protected by passive immunity, only 4% maintain anti-HBs titers during the second 6 months of life. In fact, the loss of acquired immunity, infection, and seroconversion to carrier status was reported by Dickie et al. [1981] in a child only 9 months of age in a population from the New Hebrides. A review of mother-child serological responses to HBV in this New Hebrides population led Dickie et al. [1981] to believe that acquired antibody did little to prevent eventual HBsAg(+) outcomes and, in fact, that it may promote such an outcome. Our data do not support the latter conclusion. None of the children aged 0 to 2 years and born to antibody-positive mothers were HBsAg carriers, but all four HBsAg carriers had mothers with no detectable viral markers of infection. Thus, among the Graciosa Bay Villagers of Ndeni, acquired antibody was not measurable in those 6 months of age or older and its effect was not shown to be detrimental in very young children.

**Paternal Transmission and Sexual Segregation**

Neither mothers' nor fathers' HBsAg status had a significant effect on the HBV outcome of their children aged 0 to 3 years and 3 to 5 years (see Table 1). However, when HBV outcomes of boys aged 0 to 5 years and 6 to 10 years were compared with fathers' HBsAg status, a highly significant association between fathers' HBsAg status and sons'
HBV outcome at ages 0 to 5 was found. Carrier fathers were more likely to have carrier sons (P = .002). Fathers had no measurable effect on sons aged 6 to 10 and daughters aged 0 to 5 and 6 to 10 (see Table 6).

Mothers' HBsAg status had no apparent association with the HBV outcome of children regardless of sex (Table 6). In comparing children of HBsAg(-) parents, there were relatively fewer children in the HBsAg(-) mothers group, indicating again that the small percentage of HBsAg(-) mothers limits the role of mothers in transmission of HBV to offspring. In contrast, the greater percentage of HBsAg(-) men (see Table 3) helps to facilitate the spread of HBV from father to offspring. Due to practices of sexual segregation, fathers involved in the weaning of sons, but not daughters, transmit HBV to sons and not to daughters.

Horizontal transmission by male siblings

Children 0 to 2 years old with one or more older HBsAg(+) siblings and children 0 to 2 without carrier siblings did not vary significantly in outcome. A comparison of children 3 to 5, having at least one older HBsAg(+) sibling, with children in the same age group without older carrier siblings, showed a significant difference in outcome (P = .03). At this age, children with older carrier sibs had a greater chance of becoming carriers. In analyzing the data on older siblings by sex, it was found that the HBV status of older brothers had a significant effect on the sibs ages 3 to 5 (p = .01) (see Table 7). The HBV status of older sisters did not have any measurable effect (p = .71), but only two children ages 3 to 5 had an HBsAg(+) older sister.

Strictly defined behavioral differences between the sexes enhance contacts between boys that could lead to infection. Sexual segregation rules dictate that boys eat prepared meals together and from a common bowl (30 years ago all village males ate together this way). Girls, though, are isolated and the number of contacts in food sharing is limited to female family members. This type of segregation of the sexes may help perpetuate the significant difference (.05 < P < .1) in the frequency of infection between preadolescent males and females aged 6 to 10 (see Table 3). The isolation of females also limits the effect that an infectious female has in spreading HBV. Further field observations could test these hypotheses.

Horizontal transmission of HBV between children is a significant mode of HBV infection in other societies as well. Horizontal transmission among American children in Boston as a means of infection was also shown by Leichtner et al. [1981]. In the Bos-
TABLE 7. HBsAg status of brothers and sisters compared with HBV outcome of younger sibs aged 3-5 years

<table>
<thead>
<tr>
<th>HBsAg status</th>
<th>Outcomes in children aged 3-5 years</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carrier</td>
<td>Not infected</td>
</tr>
<tr>
<td>All sisters negative</td>
<td>4</td>
<td>37</td>
</tr>
<tr>
<td>At least one sister positive</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>All brothers negative</td>
<td>3</td>
<td>40</td>
</tr>
<tr>
<td>At least one brother positive</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

1 Anti-HBe (+) and/or anti-HBc (+).

In this study, shared chewing gum was the probable vehicle of infection. On Ndeni, the sharing of food, particularly premasticated food with those who have trouble chewing, appears to be one mode of spreading infection.

As both mothers and brothers share food with young family members, it is interesting that carrier brothers, and not carrier mothers, spread HBV to children aged 3 to 5. While behavior may be the main factor affecting these results, age-related differences in the frequency of infectious carriers may also be an important factor. The frequency of carrier boys is similar to the frequency of carrier women of marriageable age. Twenty-three of 183 boys (13%) aged 3 to 15 tested HBsAg (+), and 17 of 144 women (12%) aged 21 to 50 tested HBsAg (+) (see Table 3). Married women (those generally older than 20) have a much lower proportion of infectious HBeAg (+) carriers than unmarried carrier boys (see Table 2). The four-fold difference in infectious carriers may facilitate the spread of HBV from older brother to younger sib but not from mother to child.

Homosexual practices and transmission

Among teenage boys who cohabit, the outcome of carrier is associated with having an HBsAg (+) sib. While carriers represent 11% (Table 3) of the total population of villagers, 50% of the carrier boys in cohabitation units have carrier brothers at home. A comparison with sisters' HBsAg status revealed that 67% of cohabiting boys who are carriers have carrier sisters. Thus, on Ndeni there was no significant difference in the proportion of those either infected with HBV or positive for HBsAg when young cohabiting men were compared with sibs that had no history of cohabitation. Perhaps the limited number of sexual contacts in a Graciosa Bay Village (average population < 100) accounts for the apparent ineffective spread of HBV via homosexual contact.

The similarity in HBsAg marker prevalence between boys who cohabit and their sibs suggests an additional explanation for the lack of apparent HBV infection through homosexual contact. Most males on Ndeni are infected as children long before sexual cohabitation begins. This explanation is supported by the family clustering of HBV among sibs as reported by Mazzur and Jones [1977b] using data collected in 1974 from this population.

This pattern strikingly contrasts the spread of HBV through homosexual practices in New York City. In the study of New York City homosexuals [Szmuness et al., 1975], 37-51% of male homosexuals exhibited a serological marker of HBV infection. This level of infection is at least five times greater than that in the general population (7%). Sexual activity among homosexuals was correlated with infection, and the greater the number of sexual contacts, the greater the infection rate.

Heterosexual practices and transmission

There was no apparent association between carrier husbands and infection in their wives (P = .35). Similarly, the spread of HBV through heterosexual contacts, as seen in New York City [Szmuness et al., 1975], was not apparent on Ndeni. In New York City, evidence of HBV infection was found in 26% to 28% of the spouses of HBsAg (+) individuals, while spouses of noncarriers had only a 10% to 11% rate of HBV marker prevalence.

The relatively low general prevalence of infection in New York City decreases the chances of childhood infection through family contacts. As a result, both homosexual and heterosexual contact become significant factors in the spread of HBV. Conversely,
children on Ndeni are at high risk for HBV infection, and by age 15 most individuals have been exposed to HBV. As a result, sexual contacts on Ndeni are not as significant a factor in HBV infection as they are in New York City.

**Sex-related differences in HBsAg frequency**

One interesting feature of HBV marker distribution on Ndeni is the striking increase in HBsAg (+) men over 30 years old. While 11% of 21–30-year-old men are HBsAg (+), 22% or more of men over 30 are HBsAg (+). Among those older than 30, the prevalence of HBsAg among males is significantly higher than among females [Mazzur et al., 1981].

This higher frequency in older males may be a cohort effect and may not be seen in 30 years when the boys in our study become men. The increased prevalence of carriers among older men could be related to the more traditional life-style that once existed. Post-World War II government rules, while met with resistance, did destroy the most important traditional men’s institution, the men’s house. This men’s house of the past, unlike World War II government rules, while met with resistance, did destroy the most important traditional men’s institution, the men’s house. This men’s house of the past, unlike today’s vestige, was a place where all village men gathered, sometimes bringing young sons.

Food sharing and homosexual encounters among very young boys and adults were probably responsible for an early and more widespread exposure to HBV among boys. Girls, as today, were largely unaffected by men’s house activities. As early exposure to HBV seems more likely to result in an HBsAg (+) response, the men’s house of old may have institutionalized the exposure of very young males to HBV and the subsequent development of the carrier state.

This study has focused on behavior to elucidate routes of HBV infection in a specific population. As behavior is mediated by culture, populations with different cultures have different practices. When these practices involve the spread of HBV infection, it is likely that effective routes of disease spread will differ between cultures. These differences are evident when factors such as age, sex, and family interaction are compared with regard to HBV marker prevalence. This study has determined routes that are effective in the spread of hepatitis B in Graciosa Bay Village society. Other routes of infection exist, and in other populations, some of these are the routes that spread HBV most efficiently. It is only by analyzing HBV in many populations that we can determine the dynamics of its infection.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


A. LANGENDORFER ET AL.


HEPATITIS B VIRUS

Insects
AUSTRALIA ANTIGEN IN MOSQUITOES.
FEEDING EXPERIMENTS AND FIELD STUDIES

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and W. Thomas London

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ABSTRACT

Individual mosquitoes trapped in the field in Africa and mosquitoes
studied in a laboratory were tested for Australia antigen (Au) by radioimmuno-
assay. Anopheles stephensi fed on blood containing Au had detectable Au up
to 48 hours after feeding. Ten of 42 mosquitoes trapped in residential areas
in East and Central Africa had detectable Au. In addition, one collection of
eggs from Anopheles stephensi which had fed on blood containing Au was found
to have Au. These findings support the hypothesis that mosquitoes could be a
vector of Australia antigen and hence hepatitis virus for man. They suggest
an additional hypothesis that Au could be maintained in mosquito populations
by way of maternal transmission.

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After finding that Australia antigen (Au) is related to the etiologic agent of "viral hepatitis" we suggested that it might be transmitted by mosquitoes (Blumberg et al., 1967, 1970). There have now been several reports on Au and insects (Prince et al., 1972; Smith et al., 1972; Skinhøj, 1972; Zebe et al., 1972) and in this paper we describe two kinds of mosquito studies:

1. *Anopheles stephensi* were fed on blood containing Australia antigen and blood without it. Using the radioimmunoassay (RIA) method (Coller et al., 1971), Au was found in the mosquitoes which fed on the Au positive blood, but not in the other exposed insects, nor in other controls.

2. Au was found in mosquitoes collected in several areas of East and Central Africa where Au frequencies are high.

Taken together, these studies indicate that 1) mosquitoes can carry Au; 2) Au can be detected in single mosquitoes using the RIA method; and 3) mosquitoes captured in the field carry Au. These findings are compatible with the view that mosquitoes could be responsible for the spread of hepatitis in some places.

### MATERIALS AND METHODS

**The Rat Tail Technique for Feeding Mosquitoes.**

Mosquitoes will not feed on blood unless it is presented to them in an acceptable form. The rat tail technique is suitable for feeding experiments with several species of mosquitoes (Sudia, 1971).

A laboratory white rat was killed with chloroform. While the carcass was still warm, a circular incision was made at the basal end of the tail and the tail skin loosened from the muscle by cutting the fascia. Uniform pressure was applied with the fingernails (surgical gloves were worn throughout the procedure), and the tail skin pulled slowly off the muscle. The tip of a
Pasteur pipet was cut about 1/4" below the constriction, and the rough end fire polished. After cooling, it was inserted into the upper end of the tail skin and the tail fastened with a rubber band at the point of the glass constriction. The tail was flushed with physiological saline to clear it of any loose tissue or blood.

To facilitate the immediate intake of blood by the mosquito, the whole blood was mixed with an equal part of 5% dextrose in distilled water. The mixed blood was then put into the tail skin by using a syringe with a spinal needle canula attached so as to reach the tip when filling. Once the tail skin was filled, the wide end of the glass was stoppered with a cork. The tail was placed in a Petri dish in an incubator at about 37°C for 15 minutes to warm the blood to body temperature and then placed in the mosquito cage for feeding.

Bionomics and Distribution of Anopheles stephensi Liston.

This brief description is abstracted from Foote and Cooke (1959).

Anopheles stephensi is divided into two sub-species — Anopheles stephensi mysoreasis, which is found only in India, and Anopheles stephensi stephensi, which has a more cosmopolitan distribution. Adults of Anopheles stephensi are commonly found in houses, cowsheds, barracks, and other types of manmade shelters. They feed readily on man in the laboratory and in nature. They are usually difficult to find because they hide in small, out-of-the-way places, such as the many small, dark creases and crevices in folded or crumpled cloth. Their flight range does not usually exceed one-half mile.

Larvae are found in both urban and rural locations. The outstanding type of breeding place consists of wells and other artificial containers, such as cisterns, water collections near buildings, flooded cellars, and bases of running fountains. In rural areas larvae are found in all sorts of breeding
places containing fresh, brackish, or sewage-contaminated water either in
direct sun or in the shade. Pools, stream beds, slowly moving creeks, irrigation
channels, drains, and miscellaneous breeding places with fresh water
often contain larvae. Larvae habitually sink deep into the water and stay
submerged for long periods.

The recorded distribution includes the following locations: Afghanistan,
Arabia, Assam, Bangladesh, Burma, China (South), India, Indochina, Iran, Iraq,
and Pakistan.

Specimen Preparation and Radioimmunoassay.

Individual mosquitoes were placed in tissue grinding vessels containing
0.25 ml of phosphate (0.01 M) buffered saline pH 7.3. The mosquitoes were
homogenized using a matched grooved "Teflon" pestle. Fifty µl of suspension
was drawn by micropipette for assay (Coller et al., 1971). Each sample was
run in duplicate and compared with 4 normal human sera run in duplicate. In
addition, individual unfed pooled mosquito controls were assayed for compar-
ison. There was no difference between the normal human sera and normal mos-
quitos controls. Results are expressed as number of standard deviations
inhibition from normal human serum controls.

Feeding Experiment Design.

Anopheles stephensi mosquitoes were obtained from a laboratory bred
colony at the Naval Medical Research Institute, Bethesda, Maryland. The
experiments were begun when the mosquitoes were four days old (four days
beyond the larval stage).

Two series of feeding experiments were done. The first was designed to
see if Au could be detected in single mosquitoes and the duration of Au in the
mosquito. The insects were all allowed to feed on Au(1) blood. Some were
then killed immediately after feeding and others at 24, 48, 72 and 96 hours after feeding. The individual mosquitoes were assayed for Au using the RIA method. As a control, several unfed mosquitoes were tested at the same time. This experiment demonstrated that Au could be detected and also gave information on the duration of persistence of Au in the mosquitoes.

In the second experiment, the experimental insects were allowed to feed on Au positive blood and the controls on Au negative blood.

Whole blood was collected from a patient with Down's syndrome who had Au in his blood. At the same time blood was collected from an apparently normal person who on repeated testing did not have Au. These were coded and placed in the rat tail for feeding as described above. One group of mosquitoes were allowed to feed on normal coded specimen, and the second group on the Au positive specimen. Blooded females were then placed in individual test tubes with a cotton plug moistened with sugar water. Groups of mosquitoes were killed directly after feeding (0 hours) and at 12, 24, 48, 72, 96, 115, and 137 hours after feeding. Individual mosquitoes were then prepared and analyzed as described above.

Some of the tubes contained eggs after 72 hours, which were also frozen, prepared and analyzed in the same manner as the mosquitoes. Forty-one collections of eggs were made from different tubes. In addition 10 normal mosquitoes which were not fed were prepared and tested.

All specimens were tested using coded numbers.

Field Studies.

Mosquitoes were collected in CDC mosquito light traps. A small light bulb was used to attract the insects who were drawn into and retained in the trap by means of a down-blowing fan blade driven by a small electric motor.
Compressed CO₂ (dry ice) was suspended alongside the trap as another inducement to the insects to enter. Unless otherwise mentioned, the traps were hung from low branches of trees or bushes for each of the collections.

Field collections were made in the following locations. All of these are in Uganda with the exception of 11 and 13 which are in Ethiopia.

1. Murchison Falls National Park, Chobe region. Collections were made adjacent to the residential area and within 1/8 mile of the staff quarters.

3. Murchison Falls National Park, Para region. Collections were made in a low bush area adjacent to the residential section.

7. Semiliki Forest (Rift Valley). Collections were made in low bush adjacent to the tented camp area.

8. Semiliki Forest (Rift Valley). Collections were made within the staff village.

10. Kampala. Mosquitoes collected inside a house in a middle income residential area.

11. Addis Ababa, Ethiopia. Collections were made in the compound of a residence on the Burundi Residence road.

13. Addis Ababa, Ethiopia. Collections were made in the forecourt of the Lideta Mother and Child Health and Training Center which is located in a densely populated residential area in the Lideta section of Addis Ababa.

RESULTS

Feeding Experiments.

In the first feeding experiments (Table 1) Australia antigen was detected in all of the mosquitoes which had been fed on blood containing Au and which were sacrificed at the time of feeding and after 24 hours. At 48 hours the Au levels were positive in three of the five insects tested,
Table 1. Au levels (measured by RIA) in mosquitoes fed on blood containing Au immediately after (0) and at 24, 48, 72 and 96 hours after feeding. The levels are expressed in standard deviations from the mean of duplicate assays of four normal human sera. From experience with the testing of human sera we have found that levels greater than -5 are reliably positive and are so scored in this experiment. The positive mosquitoes are underlined. The controls consist of unfed mosquitoes sacrificed at the time indicated. Two males sacrificed at 96 hours are also considered to be controls.

<table>
<thead>
<tr>
<th>Time - Hrs. after feeding on blood containing Au</th>
<th>Number of mosquitoes tested</th>
<th>Au levels in each mosquito (Standard Deviations)</th>
<th>Median</th>
<th>Number of mosquitoes tested</th>
<th>Au levels in each mosquito median</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>-20, -19, -57, -18, -53, -20</td>
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<tr>
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<td>-5, -1, +1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>8</td>
<td>-3, -5, -2, -2, -3, -4, -4, +1</td>
<td>-3</td>
<td>2 (males)</td>
<td>-2, -6</td>
</tr>
</tbody>
</table>
while none of those tested at 72 and 96 hours were positive. The levels of Au were lower at 48 hours than at 0 and 24 hours. The controls (unfed mosquitoes) at 0, 72, and 96 hours were all negative.

From this experiment we concluded that Au could be detected in mosquitoes who fed on Au. Further, the Au persisted for up to 24 hours in all the mosquitoes tested and in up to 48 hours in most. The levels in the non-fed controls were all 5 or below and scored negative, with the exception of one male insect (which presumably would not have fed on the blood) which had a level of 6.

The results of the second feeding experiment are shown in Table 2 and Figure 1. The number of positive (i.e., greater than -5 S.D.) and negative mosquitoes are shown for the indicated times of sacrifice. Insects in the first 2 rows were fed blood containing Au, and those in the third and fourth rows, blood which did not contain Au. There were four other kinds of controls. Some of the mosquitoes exposed to blood containing Au, and to the blood which did not contain Au, did not feed (rows 5 to 8). There were several males sacrificed at the 0 and 12 hour times, and there were 10 mosquitoes not allowed to feed.

In Figure 1, the levels (in S.D.) for all the mosquitoes sacrificed at 0, 12 and 24 hours are shown, as well as the levels for the mosquitoes which did not feed, and/or the mosquitoes which were not exposed to blood.

A majority of the mosquitoes sacrificed at 0, 12 and 24 hours which had fed on blood containing Au were positive for Au, while none of the other mosquitoes had positive levels. The levels in the positive mosquitoes were much higher than the controls (Figure 1). The levels at 24 hours were less than at 0 hours.

The second experiment confirms the first, with the additional control of the insects fed on blood which does not contain Au. Positive values were not
Table 2. The number of mosquitoes scored positive (> 5 S.D.) at the indicated times after feeding on blood either containing or not containing Au are shown in rows 1-4. The results for various other controls are shown in the remaining rows. For example, at 0 hours 3 of the mosquitoes fed Au + blood were positive and 1 was negative. Both of the mosquitoes fed Au negative blood were negative.

<table>
<thead>
<tr>
<th>Test Result</th>
<th>Hours after Exposure to Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Fed blood containing Au</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Fed blood which did not contain Au</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Did not feed on blood containing Au</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Did not feed on blood which did not contain Au</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Males in cage with rat tail containing Au positive blood</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Males in cage with rat tail containing Au negative blood</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Mosquitoes not fed</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

* One sample of eggs which were collected from the tube of insects fed blood containing Au and sacrificed after 72 hours were found to contain Au (-9 S.C.).
Australia antigen was detected only in mosquitoes fed on positive blood. Mosquitoes tested 48, 72, 96, 115, and 137 hours after feeding were negative for Au.

* SD = Standard deviation from the mean of 8 sera negative for Au.
Table 3. Results of the field studies. The number and short description of the stations at which collections were made are given in the first two columns. All but the last two places are in Uganda. The levels of Au found in individual mosquitoes are given in the last column. Mosquitoes scored as positive are underlined.

<table>
<thead>
<tr>
<th>No.</th>
<th>Station</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Murchison Falls, Chobé</td>
<td>-3</td>
</tr>
<tr>
<td>3</td>
<td>Murchison Falls, Para</td>
<td>-3, -3, -3, -4, -5, -2</td>
</tr>
<tr>
<td>7</td>
<td>Semiliki Forest</td>
<td>+2, +2, -4, -10, -3, -2, -3, -1, -3, -3, -5, -5, -4</td>
</tr>
<tr>
<td>8</td>
<td>Semiliki Forest, staff</td>
<td>-4, -3, -3, -8, -6</td>
</tr>
<tr>
<td>10</td>
<td>Kampala</td>
<td>-6, -4, +4, -10, -5</td>
</tr>
<tr>
<td>11</td>
<td>Addis Ababa, Ethiopia</td>
<td>-8, -5, -8, -5, -6</td>
</tr>
<tr>
<td>13</td>
<td>Addis Ababa, Lideta</td>
<td>-2, -7, -7, -2, -4</td>
</tr>
</tbody>
</table>
found after 24 hours; in this study the antigen appeared to persist for a shorter time than in the first experiment where positives were seen up to 48 hours.

One of the egg collections contained detectable Au at a level of -9 S.D. This specimen was collected from the tube containing the insects which had been fed blood containing Australia antigen and sacrificed after 72 hours.

Field Studies (Table 3).

Ten of the 42 mosquitoes tested had Au at a level greater than 6 S.D. Three were collected in the Semiliki Forest, 2 in Kampala, and 5 in Addis Ababa. All positive mosquitoes belonged to the genus Culex.

DISCUSSION

From the feeding experiments we can conclude that A. stephensi mosquitoes can carry Au and that the presence of Au can be detected in single insects. Au can be detected up to 48 hours after feeding; the levels in the insects decrease with time.

Mosquitoes caught in the wild have also been found to have detectable levels of Au; these were in general low. This might have been expected since, on the average, it is unlikely that the mosquitoes would have been trapped directly after biting a person with Au in his or her blood.

These and the previously published articles on the relation of Australia antigen and insects now make possible the investigation of a variety of interesting questions. Are mosquitoes responsible for the transmission of hepatitis and/or the Au carrier state in some communities? [See, for example, Blumberg et al. (1973), Australia antigen in the Solomon Islands, in which the high frequency of Au in some communities is compared to malaria rates.]
Is this reflected in seasonal patterns of infection? What species of mosquitoes carry Au?

Some species of non-human primates can carry Au (Blumberg et al., 1971). Do they or other animal species act as intermediate hosts in nature? Do the insects serve as mechanical transporters of Au, or can the "organism" multiply in the insects? The data from this study do not suggest growth in the insects, since the amount detected decreases with time. However, it may be difficult to detect production of Au in short term studies. Long term feeding experiments which test the hypothesis that Au can replicate in mosquitoes are in progress.

Elsewhere we have discussed the relation of Au to the genetics of the host. Do these flying insects have any role in the propagation of genetic "information"? Au was detectable in a single collection of eggs. Studies of eggs and generations of mosquitoes born to mosquitoes fed blood containing Au have been initiated. There is evidence that Au can be transmitted maternally in humans (Blumberg, 1972) and this may also be the case in certain species of mosquitoes.

Acknowledgements

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The authors also wish to express appreciation to Dr. Richard Beaudoin and staff at the Naval Medical Research Institute, Bethesda, Maryland for supplying the Anopheles stephensi mosquitoes.
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HEPATITIS B SURFACE ANTIGEN (AUSTRALIA ANTIGEN) IN MOSQUITOES COLLECTED IN SENEGAL, WEST AFRICA

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Abstract. During July and August of 1973, 9,198 mosquitoes were collected in the Republic of Senegal. Eight species of mosquitoes were found in the collections: Culex thalassius, Culex pipiens quinquefasciatus, Culex tigripes, Culex philipi, Aedes irritans, Aedes aegypti, Anopheles gambiae, and Mansonia sp. Specimens were sorted by biological condition; those obviously engorged were designated as (E), females with swollen abdomens not conspicuously blooded were considered gravid (G), and those with normal or shrunken abdomens were considered neither blooded nor gravid (U). Representative samples of each species were tested by solid phase radioimmunoassay for hepatitis B surface antigen (HBsAg, Australia antigen). A total of 12 mosquitoes were found to be HBsAg positive out of 1,658 individuals tested. These were: 9 Culex thalassius, 1 (E), 5 (G), 3 (U); 2 Culex pipiens quinquefasciatus, 1 (E), 1 (U); and Aedes irritans, 1 (U).

It has been suggested that hepatitis B surface antigen (HBsAg) may be transmitted by blood-sucking arthropods in parts of the world where both the agent of hepatitis B and such insects are common. Mechanical transmission by the contaminated proboscis of the insect is thought to be likely but has not been demonstrated in the laboratory. The experimental studies to determine whether HBsAg is replicated within a mosquito have been ambiguous. Several groups of investigators found that certain species of mosquitoes fed on blood containing HBsAg had detectable antigen for 48 to 90 hours after feeding. Their interpretation of these observations has been that HBsAg disappears as the blood is digested and that the antigen is not replicated. There are, however, some studies which are contrary to this interpretation. Smith et al. found HBsAg for 10 days in Culex pipiens which had been fed on blood containing HBsAg. The HBsAg became undetectable, but after 3 weeks was found in the salivary glands. Muniz and Micks found that HBsAg persisted for up to 6 days after feeding in Aedes aegypti. Metselaar and colleagues in East Africa found HBsAg in A. aegypti for as long as 6 weeks after feeding.

Only three previous field studies on mosquitoes have been conducted in areas of Africa with high frequencies of HBsAg in the human population. In two of these, mosquitoes of the same species were pooled, as is the practice in arbovirus studies. Prince and his co-workers found 28 of 187 pools (8 species) positive for HBsAg by radioimmunoassay, while Brotman et al. reported 17% of their collection to be positive. They suggested that replication of the antigen does not occur in mosquitoes. Since the mosquitoes were pooled, it is quite likely that mosquitoes which were not fed or gravid, but which contained HBsAg, would be overlooked. The small amount of HBsAg present in these mosquitoes might not be detected if other members of the pool did not contain HBsAg. In field collections made in Uganda and Ethiopia, we detected HBsAg in Culex species mosquitoes. In those studies, mosquitoes were tested singly but they were not identified as to species or biological state. In this paper we report on additional field studies undertaken in Senegal, West Africa in July and August, 1973. The purpose of this study was to determine: 1) whether HBsAg could be detected in wild-caught mosquitoes that were neither engorged nor gravid;
HBsAg in mosquitoes collected in Senegal

Table 1: Mosquito species collected in Senegal

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Collection site</th>
<th>Dakar*</th>
<th>Pekine</th>
<th>Pakala</th>
<th>M’Hour</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culex thalassius Theobold</td>
<td></td>
<td>117</td>
<td>796</td>
<td>260</td>
<td>5,544</td>
<td>6,717</td>
</tr>
<tr>
<td>C. pipiens quinquefasciatus Say</td>
<td></td>
<td>137</td>
<td>8</td>
<td>98</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>C. pipiens Grandpre &amp; Charmoy</td>
<td></td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>C. philippi Edwards</td>
<td></td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Aedes aegypti (Linnaeus)</td>
<td></td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Aedes iritan Theobald</td>
<td></td>
<td>-</td>
<td>6</td>
<td>12</td>
<td>2,075</td>
<td>2,093</td>
</tr>
<tr>
<td>Anopheles gambiae Giles</td>
<td></td>
<td>4</td>
<td>11</td>
<td>83</td>
<td>18</td>
<td>116</td>
</tr>
<tr>
<td>Mansoria sp.</td>
<td></td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>262</td>
<td>824</td>
<td>465</td>
<td>7,647</td>
<td>9,198</td>
</tr>
</tbody>
</table>

* Leprosarium.

2) which species of mosquito contained detectable HBsAg; and 3) the frequency of HBsAg in wild-caught specimens of the affected species.

Materials and Methods

Mosquito collections were made using CDC light traps with a modified electrical system. Dry Ice was used as an attractant. (Dry Ice was flown from Paris to Dakar each Monday morning.) Mosquitoes were killed by freezing in Dry Ice and placed in plastic vials with plastic stoppers, taped shut, marked and stored in Dry Ice. Engorged specimens were removed from the traps and stored in separate vials. All specimens were returned to the Pasteur Institute in Dakar where they were stored in a Revco freezer at -70°C. At the termination of the collections, the mosquitoes were stored in Dry Ice, carried to Paris, and then shipped by air from Paris to the Institute for Cancer Research in Philadelphia. In Philadelphia, the samples were stored at -30°C in a mechanical freezer. Using a chill table the mosquitoes were sorted by morphological characters and the species were identified. They were then stored again at -30°C until they were prepared for testing by solid phase radioimmunoassay. Care was taken to prevent the transmission of hepatitis or any exotic arbovirus to laboratory workers. All identifications were confirmed by the Smithsonian Institution, Southeast Asia Mosquito Project, Washington, D.C.

Solid Phase Radioimmunoassay

Individual mosquitoes were ground in 250 µl of cold fetal calf serum (Grand Island Biological Co.) with the aid of a 4.5-ml capacity ground glass surfaced tissue grinder. Ground mosquito suspensions were maintained in an ice bath and tested immediately or frozen at -30°C until assayed. The solid state assay system used was a modification of the technique reported by Purcell et al. In our system, dissociated human antibody to HBsAg (anti-HBs) was used to coat disposable microtiter V well plates. The IgG fraction of guinea pig anti-HBs, isolated by DEAE cellulose fractionation, was labelled with 125 I by the procedure of Greenwood et al. and used in the final application of the “sandwich” technique. Each sample was assayed in duplicate and in several cases in quadruplicate. Each assay plate contained at least 10 controls. Controls included ground non-fed mosquitoes, mosquitoes fed normal human serum, mosquitoes fed guinea pig serum and normal human plasma. The control mosquitoes were laboratory reared Culex pipiens quinquefasciatus. None of the controls was found to differ significantly. A mosquito was considered positive only if duplicate determinations were greater than 1.5 X the mean of the 10 controls and if repeat determinations in duplicate were also greater than 1.5 X the mean of the controls.

Mosquito Species

Table 1 lists the species identified and locations of each collection. Aedes irritans and Culex thalassius represent 90% of the collection. Their large representation reflects the serious drought in these regions over the past 5 years. Aedes irritans and Culex thalassius breed in salt marshes and crab holes along the ocean. Culex pipiens quinquefasciatus was not as abundant as we had expected, particularly in light of the poor sanitary conditions.
in the area. (Few Culex pipiens were found either in the light traps or in collections with battery operated aspirators made in villages.) This cosmopolitan mosquito is thought to have been introduced into West Africa only within the last 200 years. Aedes aegypti was not readily attracted to the traps, although larvae were abundant in certain areas in cisterns and artificial containers. Anopheles gambiae did not seem to favor the trap, and most of the adult females were collected with a battery-run mechanical hand aspirator in native huts in the village of Pakala. Although Culex phillipi is also a salt marsh mosquito, only one specimen was collected in a light trap at the leper hospital adjacent to the University of Dakar.

**Collections**

Four areas were chosen for collection sites (Table 1).

1) **Leprosarium, Dakar.** This area was a small leper hospital with about 40 beds, adjacent to the University of Dakar. Trap collections were relatively unproductive from this area (Table 1) since the collections were made at the end of July and the first week of August before any significant rain had fallen. Although close to the ocean, there was little opportunity of brackish water species to breed due to the topography of the terrain. A leprosarium was selected for this collection since it is known that there is a high frequency of HBVAg in lepromatous leprosy patients.

2) **Pekine.** Pekine is a small suburb about 12 kilometers east of Dakar. It was selected as representative of a densely populated residential area similar, in general, to many others in the Dakar area.

3) **Pakala.** Pakala is a village 10 kilometers east of the town of Thies and about 65 kilometers from Dakar. Collections were made on the night of the 8th of August. There was a heavy storm this night which caused a malfunction of the light bulbs on two of the traps. It was relatively easy, however, to use the mechanical aspirator and a flashlight to collect Anopheles gambiae on the inside walls of the huts.

4) **M'Bour.** This small town along the coast 45 kilometers south of Dakar was by far the most productive area for mosquitoes. The collection site was approximately 1.5 kilometers from a leper village.

**RESULTS AND DISCUSSION**

Out of a total collection of 9,198 specimens, 1,648 were tested individually by solid phase radioimmunoassay for HBVAg. The mosquitoes tested by the radioimmunoassay method (Table 2) were selected as follows. For those species of which only a small number was caught (C. pipiens quinquefasciatus, A. aegypti, A. gambiae), all specimens in good condition were tested. However, for three species (C. tigripes, C. phillipi, Mansonia sp.) no specimen was tested since only a few were collected and they were used for identification. Specimens used for identification were subsequently deposited with the Smithsonian Institution. A 10% sample was taken of the two most common species (C. thalassius, A. irritans).

Of 1,088 specimens of C. thalassius, 9 were found HBVAg positive (Tables 2 and 3). Of those positive, 1 had recently had a blood meal. 5 were gravid, but 3 had neither recently fed nor were they gravid. Two hundred and three C. pipiens quinquefasciatus were tested, and 2 were found to be positive. Of those, one was engorged but
IIU AG IN MOSQUITOS COLLECTED IN SENEGAL

Results of solid phase radioimmunoassay for hepatitis B surface antigen, showing biological condition of mosquitoes and minimum field infection rate (MFIR)

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Blooded</th>
<th>Gravid</th>
<th>Neither blooded nor gravid</th>
<th>MFIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culex thalassius</td>
<td>78 (1)*</td>
<td>398 (5)</td>
<td>612 (3)</td>
<td>1:204</td>
</tr>
<tr>
<td>C. pipiens quinquefasciatus</td>
<td>3 (1)</td>
<td>-</td>
<td>200 (1)</td>
<td>1:200</td>
</tr>
<tr>
<td>Aedes irritans</td>
<td>4 (0)</td>
<td>82 (0)</td>
<td>226 (1)</td>
<td>1:226</td>
</tr>
</tbody>
</table>

* The number of mosquitoes positive for Australia antigen is shown in parentheses.

The second was neither engorged nor gravid. The only other positive mosquito was 1 of 226 Aedes irritans tested and it also was neither engorged nor gravid.

As previously mentioned, HBAg has been detected up to 72 hours and longer after experimental feedings but prior to this study has not been demonstrated in non-engorged, non-gravid wild caught female mosquitoes.

The minimum field infection rate (MFIR) for the three species in which HBAg was found was approximately 1 in every 200 mosquitoes (Table 4).

Arthropod-borne diseases have the following characteristics. The infectious agent is found in the blood of an infected vertebrate host. The agent can be transmitted from the host to an arthropod in a blood meal. The arthropod can transmit the agent to another host. As strictly defined, the agent will replicate in the vector, but simple mechanical transmission by arthropods may be sufficient. HBAg is found in the peripheral blood of patients with hepatitis and of hepatitis carriers. It has been demonstrated in mosquitoes which are gravid, which have recently fed, and in mosquitoes which are neither of these. Experimentally, it has been shown that mosquitoes of several species can imbibe HBAg in blood and that it can be detected in the mosquito for several days or longer after feeding. Except for the report of Smith et al., it has not been shown that HBAg can be transmitted from the insects to experimental animals, nor is it known whether the hepatitis B virus (HBV) can replicate in the mosquito. If either of these is demonstrated, then HBV could be considered an arthropod-borne infectious agent. Further, the field infection rate of non-engorged, non-gravid mosquitoes is similar to that of mosquitoes which transmit other arthropod-borne viruses. The agent appears to be present in a large number of mosquito species.

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HEPATITIS-B VIRUS IN BEDBUGS (CIMEX HEMIPTERUS) FROM SENEGAL

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Summary
Bedbugs of the species *Cimex hemipterus* (F) were collected on four separate occasions from the bedding in the huts of village dwellers in Senegal, West Africa. Hepatitis-B surface antigen (HBsAg) was detected in unengorged nymph and adult bedbugs in each of the first three collections. 3 of 28 such specimens were HBsAg(+) in the first collection and 3 of 17 specimens were positive in the second collection. In the third, 6 of 9 were HBsAg(+) when the bed occupant was known to be HBsAg(-). 2 of these 6 positive insects did not contain human serum proteins. Bedbugs in the fourth collection were captured and kept alive without a blood meal for 30 days. 3 of 89 of these samples were HBsAg(+). These are the highest field infection-rates of hepatitis-B virus reported in any insect species. The bedbug must be considered a potential vector of hepatitis-B virus.

Introduction
Blood-sucking arthropods have been proposed as vectors of hepatitis-B virus (H.B.V.). In tropical and other areas most of the population may show evidence of present or past infection with H.B.V., and in such areas lifetime exposure to mosquitoes and other arthropods is frequent. Many workers have studied mosquito species, and field infection-rates—i.e., the prevalence of hepatitis-B surface antigen (HBsAg) in unengorged, non-gravid mosquitoes—have ranged from 1 in 200 in several species to 1 in 100 in *Anopheles gambiae*. Other blood-feeding insects such as bedbugs, which live more intimately with man than do mosquitoes, have not been evaluated systematically as potential vectors of H.B.V. We know of two previous efforts to assess the role of HBsAg in bedbugs. Brotman et al. collected bedbugs from the beds of prostitutes in the Ivory Coast. They pooled their specimens and found one of eighteen pools to be positive for HBsAg. Newkirk et al. in laboratory experiments, fed the temperate bedbug *Cimex lectularius* (L) on HBsAg(+) blood. They found that HBsAg persisted in these insects for 35 days or more after feeding. In some instances the titres of antigen appeared to rise after the disappearance of human serum proteins, suggesting replication of the virus.

Here we report high field infection-rates of H.B.V., as detected by the presence of HBsAg, in unengorged bedbugs collected from the beds of inhabitants of villages in Senegal, West Africa.

Materials and Methods
Bedbugs were collected from huts in rural villages in Senegal on four occasions. Collection A was made during the rainy season in August, 1975, from three villages near Thies, about 70 km east (inland) of Dakar; collection B was made from the same three villages in the dry season in March, 1976; collection C was made in the village of Dieng, near Djurbel, about 170 km east of Dakar in September, 1976; and collection D was from the same village in November, 1976.

The bedbugs were captured by searching bedding during the daytime, using a flashlight. Bugs from each bed were placed in a separate container. On the first three occasions, the insects were killed by freezing a few hours after capture, and kept at −30°C or colder. These bugs were returned to the U.S.A. on dry ice and stored in a freezer until they were tested. Bugs of collection D were captured and returned alive to the U.S.A. (Cimex is free from U.S.A. quarantine restrictions) where they were maintained at 28°C for 30 days in an insectary at Harrisburg, Pennsylvania, when they were killed and stored in the freezer.

While still frozen the bugs were examined under a stereomicroscope and their stage of development (nymph or adult), sex (of adults), and state of engorgement were noted. They were then ground in a 0.25 ml (or 0.50 ml for bedbugs in collection D) solution of 1% human serum albumin with 0.01% merthiolate preservative, using glass-in-glass tissue grinders, as previously described. The homogenates were frozen immediately. Later, without further processing, they were thawed and tested in duplicate by radioimmunossay ('Austria II-125', Abbott Laboratories, North Chicago, Illinois). The criteria of positivity were those recommended by Abbott Laboratories. Other arthropods which we have tested (e.g., ticks, fleas, and unfed mosquitoes) were all HBsAg(−) in this assay.
In collection C only, the homogenates were tested for human serum proteins (other than albumin) by immunoelectrophoresis in 1-1% agarose gel using polyvalent antihuman whole serum produced in horses (Behring Diagnostics, Somerville, New Jersey). The HB,Ag+ bedbugs of collection C were also tested for e antigen and anti-e by rheophoresis in 0.8% agarose plates (Abbott Laboratories).

**Results**

All bedbugs collected were identified as *C. hemipterus* (F), the predominant species in West Africa and other tropical areas. The results of the HB,Ag tests on bedbugs from the four collections are shown in table 1.

In 1975, we collected 28 unengorged bedbugs from villages near Thies. Of these, the 1 unengorged nymph collected was HB,Ag(+) and 2 unengorged adults out of 27 tested were HB,Ag(+). In March 1976, 48 bedbugs were collected from the same villages. Of these, 1 of 11 unengorged nymphs, 2 of 6 unengorged adults, and 3 of 20 engorged adults were positive for HB,Ag.

In August, 1976, 18 bedbugs were collected from the beds of four individuals whose status with respect to HB,Ag was known (collection C). The beds were wooden cots which stood directly on the dirt floors of the huts. Each hut contained one, two, or three beds, one of which had been occupied for more than a year by the designated occupant. We were not able to determine whether other individuals occupied the same bed transiently. 16 bedbugs were from the beds of two HB,Ag(+) individuals (a 17-year-old boy and a 12-year-old girl) and 2 were from the beds of HB,Ag(−) persons (a 40-year-old woman and 16-year-old boy). 11 of the 16 bedbugs from the antigen-positive persons were HB,Ag(+) and the 2 bedbugs from antigen-negative individuals were HB,Ag(−). Among the 11 HB,Ag(+) bedbugs were 2 unengorged nymphs and 4 unengorged adults. The prevalences of HB,Ag in engorged (5 positive of 7 tested) and unengorged specimens (6 positive of 9 tested) were similar.

**Table I—Results of HB,Ag Tests of Bedbugs Collected in Senegal**

<table>
<thead>
<tr>
<th>Collection</th>
<th>Nymphs</th>
<th>Adults</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>U</td>
<td>HB,Ag(+)</td>
<td>Caught</td>
</tr>
<tr>
<td>B</td>
<td>E</td>
<td>HB,Ag(−)</td>
<td>Caught</td>
</tr>
<tr>
<td>C</td>
<td>E</td>
<td>HB,Ag(+)</td>
<td>Caught</td>
</tr>
<tr>
<td>D</td>
<td>S</td>
<td>HB,Ag(−)</td>
<td>Caught</td>
</tr>
</tbody>
</table>

**Table II—Human Serum-Proteins* in Unengorged Bedbugs**

<table>
<thead>
<tr>
<th>Serum protein</th>
<th>+</th>
<th>−</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB,Ag+</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>HB,Ag−</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* Serum-proteins were assayed by immunoelectrophoresis. Plates were scored (+) or (−) without knowledge of HB,Ag results.
To assess whether the presence of HB$_A^g$ in the unengorged insects was a reflection of incomplete digestion of a previous blood meal, we tested all unengorged bedbugs in collection C for human serum proteins by immunoelectrophoresis, without knowledge of the HB$_A^g$ results. 2 of the 6 HB$_A^g(+)$ unengorged bedbugs did not contain detectable human serum proteins by immunoelectrophoresis, a relatively insensitive method, while the others did (table II).

All of the HB$_A^g(-)$ bedbugs were tested for e antigen, a marker of infectivity.$^{14,15}$ 1 engorged and 1 unengorged, serum-protein-positive bedbug were e-antigen positive, suggesting that these insects contained virus particles which are likely to be infectious.$^{13}$ e antigen was not detected in the serum of the HB$_A^g(+)$ individual from whose bed these 2 bugs were collected. Anti-e was not found in any of the bugs.

Collection D was made to gain further information on the role of digestion of the blood meal by the bedbugs. Field-captured insects were held for 30 days in an insectary without additional blood feeding, by which time human serum proteins should be fully digested.$^{10}$ 3 of 89 specimens were HB$_A^g(+)$.

Discussion

The field infection-rates of h.b.v. that we observed in unengorged C. hemipterus are the highest reported in any insect species. Although the presence of HB$_A^g$ or even the presence of whole virus is insufficient evidence for accepting the bedbug as a vector of h.b.v., the data are sufficient for this to be considered a possibility.

Several aspects of the epidemiology of hepatitis B are compatible with a role for bedbug transmission. Hepatitis B is frequently transmitted to sexual partners,$^{16}$ and mother-to-infant transmission is particularly common in the tropics.$^{13,18}$ Blood-sucking arthropods feeding on the occupants of the same bed could amplify the risk of hepatitis-B infection for previously uninfected persons or help perpetuate infection in infected individuals by repeated inoculations of virus.

In the past bedbugs were not considered vectors of disease, but lately two groups have observed that Cimicidae may be reservoirs and vectors of viruses in nature. Our studies of C. hemipterus suggest that this arthropod may be a vector of h.b.v. Clearly, h.b.v. antigens can persist in this organism for long periods, but whether it can transmit h.b.v. to man or laboratory animals remains to be determined.

We thank Mamadou Sarr of S.L.A.P. in Thies, Senegal, and Beatrice Tremolet and Caroline Jamet for assistance in the collections; Dr Jean Coz of O.R.S.T.O.M. at the Pasteur Institute in Dakar, Dr Yves Robin, Pasteur Institute, Dakar, and Dr de Lautrec, department of hygiene, Faculté de Médecine, for providing laboratory space and technical assistance. This work was supported by U.S. Public Health Service grants CA 06551, RR 05539, and CA 06927 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania.

Requests for reprints should be addressed to W.T.L., Institute for Cancer Research, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111, U.S.A.
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20. Monash, T. Personal communication.
HEPATITIS B VIRUS

Cancer
ANTIBODY TO HEPATITIS-B CORE ANTIGEN IN PATIENTS WITH PRIMARY HEPATIC CARCINOMA

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Summary Antibody to hepatitis-B core antigen (anti-HBc) was assayed in the serum of patients with primary hepatic carcinoma (P.H.C.) and controls from Hong Kong, West Africa, and the United States. In each region the prevalence of anti-HBc was higher in P.H.C. patients than in controls, ranging from 70 to 95% in the patients and from 20 to 68% in the controls from Asia and Africa; 24% of P.H.C. patients and 4% of controls from the U.S. had anti-HBc. These data support the hypothesis that chronic infection with hepatitis-B virus is at least one etiological factor in P.H.C., especially in Asia and Africa, although other factors must also be involved.

INTRODUCTION

Primary hepatic carcinoma (P.H.C.) is the most common malignant neoplasm in several of the world's major populations (Chinese, Africans, Filipinos). Therefore, it may be the most common cancer in man.

On the basis of necropsy studies showing an association between chronic hepatitis and/or macronodular post-necrotic cirrhosis with P.H.C., Payet and Steiner and Davies suggested that viral hepatitis may be the major cause of P.H.C. in Africa. When tests for infection with hepatitis-B virus (H.B.V.) became available it was possible to evaluate the association of H.B.V. with P.H.C. Several studies in Africa and Asia showed an increased prevalence (35-80%) of Australia antigen (hepatitis-B surface antigen, HB,Ag) among patients with P.H.C. These high prevalences were generally detected only when sensitive methods (radioimmunoassay, immune adherence hemagglutination) for assaying HB,Ag were used. This observation suggested that either HB,Ag was present in decreased quantity in P.H.C. patients or there was some antigenic difference between the HB,Ag in P.H.C. and that found in HB,Ag carriers. No increase in the prevalence of antibody to hepatitis-B surface antigen (anti-HB,) has been reported in P.H.C. patients.

If patients with P.H.C. are infected with H.B.V. but are producing small quantities of HB,Ag, there may be defects in viral assembly or viral replication in the malignant cells. Hoofnagle et al. have suggested that antibody to hepatitis-B core antigen (anti-HBc) is a better indicator of intracellular replication of H.B.V. than anti-HBc or HB,Ag. In this paper we report the detection of anti-HBc in a very high proportion of patients with P.H.C. These data support the hypothesis that chronic infection with H.B.V. is at least one etiological factor in P.H.C. and suggest a method of preventing this form of cancer.

MATERIALS AND METHODS

HB,Ag was extracted by the method of Hoofnagle et al. from the liver of a patient who had chronic hepatitis, was positive for HB,Ag, and had received a renal transplant. Electron microscopy of a biopsy specimen of this patient's liver showed numerous 27-29 nm. particles in the nuclei of parenchyma cells. (Attempts by the methods described below to extract HB,Ag from the livers of four other HB,Ag carriers and one liver from an HB,Ag-negative person were unsuccessful.) The liver was removed at necropsy, perfused with 0.25 M sucrose, and frozen in dry ice. For preparation of core antigen the liver was thawed and homogenised in a Waring blender as a 25% suspension in cold phosphate-buffered saline. The homogenate was sonicated twice for 60 seconds each at maximum setting in a Bronson W 185E sonicator (Heat Systems Inc.) with special microtip. The procedures used to concentrate and isolate HB,Ag are shown in the figure.

HB,Ag in the liver homogenate (extract HB,B2) reacted by C.E.P. and immunodiffusion (I.D.) with the rhesus anti-HBc and the precipitin in I.D. against HB,B2. C.E.P. was carried out in 1.1% agarose in barbitone buffer, pH 8.2. I.D. was performed in 0.9% Noble agar in barbitone buffer, pH 8.2. HB,B2 was placed in the centre well and known anti-HBc was placed in the first and fourth wells of a seven-well I.D. pattern. Sera were considered to contain anti-HBc if they
ANTI-HBc in Patients with P.H.C.

<table>
<thead>
<tr>
<th>Location</th>
<th>No.</th>
<th>Anti-HBc+ (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Dakar, Senegal-A</td>
<td>43</td>
<td>41 (95%)</td>
<td>2.3 x 10^-11</td>
</tr>
<tr>
<td>P.H.C. Controls</td>
<td>62</td>
<td>16 (26%)</td>
<td></td>
</tr>
<tr>
<td>(2) Dakar, Senegal-B</td>
<td>28</td>
<td>25 (89%)</td>
<td>0.055</td>
</tr>
<tr>
<td>P.H.C. Controls</td>
<td>28</td>
<td>18 (64%)</td>
<td></td>
</tr>
<tr>
<td>(3) Bamako, Mali</td>
<td>24</td>
<td>17 (71%)</td>
<td>7.8 x 10^-4</td>
</tr>
<tr>
<td>P.H.C. Controls</td>
<td>40</td>
<td>10 (25%)</td>
<td></td>
</tr>
<tr>
<td>(4) Hong Kong</td>
<td>37</td>
<td>26 (70%)</td>
<td>2.3 x 10^-4</td>
</tr>
<tr>
<td>P.H.C. Controls</td>
<td>56</td>
<td>21 (38%)</td>
<td></td>
</tr>
<tr>
<td>(5) Philadelphia, U.S.A.</td>
<td>21</td>
<td>5 (24%)</td>
<td>0.041</td>
</tr>
<tr>
<td>P.H.C. Controls</td>
<td>50</td>
<td>2 (4%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>153</td>
<td>114 (75%)</td>
<td>2 x 10^-11</td>
</tr>
<tr>
<td>Controls</td>
<td>238</td>
<td>67 (28%)</td>
<td></td>
</tr>
</tbody>
</table>

* (1) Patients with P.H.C. in Hospital Le Dantec, Dakar. Controls are 1 or 2 persons without P.H.C. from Senegal matched for each case by age, sex, and ethnic group.
(2) Patients with P.H.C. at Hospital Le Dantec, Dakar. Controls are individuals without P.H.C. matched for each case by age, sex, and ethnic group.
(3) Patients with P.H.C. at Hospital Point G, Bamako. Controls are 1 or 2 persons from Mali without P.H.C. matched for each case by age, sex, and ethnic group.
(4) Patients with P.H.C. collected by Dr David Todd at the Queen Mary Hospital, Hong Kong. Controls are 58 patients without P.H.C. from the same hospital with a variety of other diseases. 7 of the 58 controls had cirrhosis; 5 of these had anti-HBc.
(5) Sera from patients with P.H.C. were submitted by physicians at several hospitals in Philadelphia. Controls were 25 healthy volunteer blood-donors and 25 prisoner blood-donors.

† Calculated from Fisher's exact 2 x 2 test.

The table shows a line of identity with known anti-HBc by I.D. and a precipitin by C.E.P. and E.M. Sera which gave equivocal results were tested by C.E.P. against HBcAg Amicon concentrate and the gradient fractions containing HBcAg. If a precipitin was observed, such sera were scored anti-HBc positive. In general, C.E.P. and I.D. using the HBcAg fraction gave definitive results.

PATIENTS AND CONTROLS

The P.H.C. patients and controls tested for anti-HBc are listed in the table. The diagnosis of P.H.C. was accepted only if patients had histological evidence of P.H.C. on biopsy or at necropsy or if their clinical picture was compatible with P.H.C. and they had α-fetoprotein detectable in their serum by C.E.P. or I.D.

RESULTS

The table shows that, of 153 patients with P.H.C. from three continents, 114 had anti-HBc detected by the relatively insensitive C.E.P. and I.D. tests. In Senegal
over 90% of the patients had anti-HBc whereas in Mali and Hong Kong about 70% were positive. Of the 5 anti-HBc-positive American P.H.C. patients, 1 was Chinese and another was from Puerto Rico.

**DISCUSSION**

The prevalence of anti-HBc among these P.H.C. patients is higher than the reported prevalence of HBsAg or anti-HBc among P.H.C. patients. The frequency of these factors in P.H.C. patients will be reported elsewhere in studies describing the variation of response of West Africans to H.B.V. infection. Here we wish to emphasise the likelihood that virtually all P.H.C. patients in West Africa and South-East Asia are (or have been) infected with H.B.V. In the U.S.A., H.B.V. is associated with a considerable proportion of, but not all, cases of P.H.C.

Furthermore, anti-HBc must be present in high titre to be detectable by C.E.P. and I.D., whereas HBsAg is present only at low concentrations in P.H.C. patients. This suggests that in P.H.C. there is a continuous production of core antigen and limited synthesis of HBsAg. Hadziyannis has reported that, by immunofluorescent techniques, HBsAg is found only in the parts of the liver unaffected by P.H.C. On the basis of these observations and the data reported here, we suggest that both the unaffected and malignant liver cells are producing HBsAg.

The data do not in themselves indicate a causal relationship between hepatitis B and P.H.C., but, taken together with previous findings, they support the hypothesis that H.B.V. infection is aetiologically related to P.H.C. Since infection with H.B.V., as detected by the presence of HBsAg, anti-HBc, or anti-HBc, is common in the general populations of West Africa and Hong Kong, additional factors must be involved in the causation of P.H.C. Some of these are under investigation and will undoubtedly be identified in due course. No matter what other factors are involved, however, if H.B.V. infection is one necessary factor then prevention of infection with H.B.V. could lead to prevention of P.H.C. With the development of a vaccine against H.B.V., prevention of many cases of P.H.C. may be a realistic expectation.

We thank Dr S. N. Huang for supplying the human liver for the source of HBsAg, Dr David Todd for the sera from the Chinese patients and controls, Dr Jay Hoofnagle for the rhesus anti-HBc antibody, and Barbara Peticolas, Ellen Halpert, Theresa Halbherr, and Francis Kern for technical assistance.

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Requests for reprints should be addressed to W. T. L., Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111, U.S.A.

**REFERENCES**

The Relation of Infection With the Hepatitis B Agent to Primary Hepatic Carcinoma

Baruch S. Blumberg, MD, Bernard Larouzé, MD, IHP, W. Thomas London, MD, Barbara Werner, PhD, Jana E. Hesser, PhD, Irving Millman, PhD, Gerard Saimot, MD, and Maurice Payet, MD

In Asia, Africa, and other tropical areas, primary hepatic carcinoma (PHC) is associated with liver cirrhosis of the postnecrotic (macronodular) type. Chronic viral hepatitis is likely to be the cause of this cirrhosis in many patients from regions where chronic infection with the hepatitis B virus (HBV) is common. More than 95% of patients with hepatoma (in Mali and Senegal) have evidence of infection with HBV, a much higher frequency than in controls. Thirty-nine of 62 patients with PHC had hepatitis B surface antigen (HBsAg) (controls, 8 of 98) and 56 of 63 (controls, 26 of 100) had antibody against hepatitis B core antigen (anti-HBc). In earlier studies, we demonstrated a maternal effect of HBsAg. If the mother has the antigen and the father does not, the children are much more likely to also have HBsAg than if the father has the antigen and the mother does not (93/161 = 57.8% when mother is positive vs. 28/135 = 20.7% when father is positive; P = 0.6 X 10^-19). Studies in Greece and in the Solomon Islands show that presence of HBsAg in parents affects the sex ratio of the offspring of the mating. This implies that the presence of the agent in a parent can affect the fetus early in life. Parental studies in the west African hepatoma patients showed that there is a very high frequency of HBsAg in mothers (71.6%), while the frequency in fathers (18.5%) is significantly less. This suggests that the development of hepatoma in offspring is related to infection in parents. Several years ago, we described a vaccine which may be useful in preventing infection with hepatitis B. Strategies are discussed which might be effective in preventing the development of carriers with, it is hoped, a consequent decrease in the frequency of HBV carriers, chronic hepatitis, and primary hepatic carcinoma. The strategy would employ methods for decreasing the frequency of the agent in the environment by the application of public health methods including the vaccination of appropriate newborns and other members of the population. (Am J Pathol 81:669-682, 1975)

During recent years, there have been parallel developments in understanding, on the one hand, the pathogenesis of primary hepatic carcinoma (PHC, hepatoma) and, on the other, the biology of Australia antigen (hepatitis B surface antigen) and the infectious agent, hepatitis B virus (HBV), to which it is intimately related. Recently, the
paths of these developments have begun to converge, and from this it is possible to design a preliminary strategy which could, if the interpretations of these data are correct, result in the prevention of many, and perhaps most, cases of one of the most widespread and deadly cancers of humans. These developments will be discussed under five categories, and in a sixth section suggestions for the use of this information will be given. The subject headings are: a) The relation of hepatoma to hepatitis in Africa and Asia. b) The relation of HBV to hepatoma. [Hepatitis B surface antigen (HBsAg); antibody to the surface antigen (anti-HBs); antibody to hepatitis B core antigen (anti-HBc)]. c) Family clustering of HBsAg. Maternal (and possibly paternal) transmission of HBV. d) Presence of HBsAg in the mothers of patients with hepatoma. e) The development of a vaccine against HBV. f) Considerations of strategies to prevent hepatoma in areas where it occurs in high frequency.

These studies may also be useful in investigating other agents which may be related to the pathogenesis of other cancers.

The Relation of Hepatoma to Hepatitis B in Asia and Africa

Primary hepatic carcinoma is the most common form of cancer occurring in males in certain areas of the world with large populations, e.g., China, Africa, the Philippines. Thus, although PHC is a relatively uncommon disease in the United States, it is an international health problem of major significance. For example, it has been stated that in Taiwan it is the second most common cause of death.¹

Hepatoma is associated with liver cirrhosis of the postnecrotic (macronuclear) type. Hepatoma often arises in a liver affected by long standing cirrhosis (70 to 80%), but hepatoma and cirrhosis may develop simultaneously or, less commonly (10%), hepatoma may occur in an otherwise normal liver.² Since postnecrotic cirrhosis is the response of the liver to an agent (or agents) which causes necrosis of hepatic cells, much of the research on hepatoma has centered on studies of hepatotoxic agents which are common in areas where hepatoma is common. For example, considerable effort has been expended on the studies of cycads, mycotoxins, and nitrosamines. The best-documented cause of postnecrotic cirrhosis, however, is viral hepatitis. In the late 1950s and early 1960s, Payet ³ and Steiner and Davies ⁴ suggested, on epidemiologic and pathologic grounds, that viral hepatitis was the major cause of hepatic cirrhosis and hepatoma in east Africa (Uganda) and west Africa (Senegal). Since, at that time, no tests were available for identifying the viral agent of hepatitis, this hypoth-
Hepatic carcinoma and HBV

A hypothesis could not be tested adequately. With the discovery of Australia antigen and its specific association with hepatitis B, this became possible. This hypothesis states that there is a relation between infection with the hepatitis B agent, the development of chronic hepatitis and chronic liver disease, and the development of hepatoma. These relations need not be linear, and it is also obvious that other factors must be involved. Not everyone infected with HBV develops hepatitis or becomes a carrier (some develop anti-HB, and in some persons exposed to HBV the effect is not measurable). Nor does everyone who develops chronic hepatitis develop postnecrotic cirrhosis, and of those who do, even in high-incidence areas, only a fraction develop hepatoma. From this, it is obvious that other factors may also be needed to develop hepatoma. If there are many factors essential to cause an illness, the prevention of only one of them could prevent the development of the disease. Complete knowledge of all the etiologic factors is useful, but not essential, in designing prevention strategies.

In general, carriers of HBsAg are common in areas where hepatoma is common. Do some of these carriers have chronic hepatitis? There is evidence that this is the case. In Table 1 (taken from Economidou et al.6) a summary of studies on liver chemistry abnormalities in carriers is shown. In most of these locations some of the carriers have evidence of liver malfunction. In a matched pair study in the village of Plati, Greece, a high prevalence area, it was shown that there is a significantly higher level of SGPT in the carriers than in matched individuals who are not carriers.6,7

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of donors</th>
<th>Prevalence of HBsAg</th>
<th>SGPT determination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. HBsAg carriers tested</td>
</tr>
<tr>
<td>Okochi and Murakami</td>
<td>3,131</td>
<td>1.78</td>
<td>56</td>
</tr>
<tr>
<td>Soulier et al.</td>
<td>18,084</td>
<td>0.43</td>
<td>35</td>
</tr>
<tr>
<td>Otto-Servais et al.</td>
<td>6,656</td>
<td>0.15</td>
<td>9</td>
</tr>
<tr>
<td>Wewalka</td>
<td></td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>Sanwald and Sauerbruch</td>
<td>2,053</td>
<td>0.83</td>
<td>17</td>
</tr>
<tr>
<td>Klinman</td>
<td>52,429</td>
<td>0.1</td>
<td>23</td>
</tr>
<tr>
<td>Cazal and Robinet-Levy</td>
<td>52,966</td>
<td>0.28</td>
<td>40</td>
</tr>
<tr>
<td>Szmuness et al.</td>
<td>208,000</td>
<td>0.15</td>
<td>197</td>
</tr>
<tr>
<td>Economidou and Binopoulou</td>
<td>6,606</td>
<td>2.9</td>
<td>143</td>
</tr>
</tbody>
</table>

* The definition of abnormal is generally an elevation of SGPT greater than 50 IU.
† Number with abnormal tests.

Reproduced from Economidou et al.6 (Res Commun Chem Pathol Pharmacol 10:703, 1975) by permission of P.1n Publications.
From this we may conclude that a proportion of carriers in these regions have chronic liver malfunction and it is reasonable to conclude that, over the course of many years of the carrier state, their livers could be significantly affected.

The Relation of Hepatitis B Virus to Hepatoma

In the early studies of association of HBsAg with hepatoma, insensitive detection techniques were used. These showed no or weak associations of HBsAg with hepatoma. In recent years, however, sera from patients with hepatoma have been tested by very sensitive methods, immune adherence hemagglutination (IAHAA) and radioimmunoassay (RIA), and high frequencies of HBsAg among the patients with hepatoma from different parts of the world have now been found (Table 2). Although an association of HBsAg with hepatoma has not been documented in American and northern European populations, the increased frequency of HBsAg in hepatoma detected by RIA and IAHAA is not paralleled by a similar increase in detection frequency among the general population of the same area. This suggests that HBsAg in hepatoma patients is either present in small amounts or that the antigen present in patients with hepatoma is antigenically similar but not the same as HBsAg. There are other interrelationships of HBsAg, cirrhosis, and hepatoma which also suggest that these three entities may be causally interconnected. For example, all three are more common in males than females and all may cluster in families.

Recently it has become possible to test not only for HBsAg but also for anti-HBs, antibody against the surface antigen, and anti-HBc, antibody against the core antigen (HBcAg) of the hepatitis B agent. These presumably all indicate that the individual has been infected with HBV. Preliminary results of our study in Mali and Senegal are shown in Table 3 (adapted from Maupas et al. and Larouzé et al.). From this it can be said that nearly all of the hepatoma patients have some evidence of infection. The frequencies of HBsAg and anti-HBc are higher in the hepatoma patients than in controls. The frequency of anti-HBc is higher in the controls in Senegal (also true in Japan, see Table 2), suggesting that the hepatoma patients have a different biologic response to HBV than the controls.

Family Clustering of HBsAg

Among the earliest investigations of Australia antigen were studies on family distribution. The initial studies supported the hypothesis that HBsAg in carriers segregated as if it were controlled by an autosomal
Table 2—HB$_3$Ag and Anti-HB$_3$ In Primary Hepatic Carcinoma

<table>
<thead>
<tr>
<th>Location</th>
<th>PHC</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Technique</td>
<td>No. tested</td>
</tr>
<tr>
<td>Singapore*</td>
<td>CEP</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>IAHA</td>
<td>156</td>
</tr>
<tr>
<td>Japan*</td>
<td>IAHA</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>IAHA</td>
<td>86</td>
</tr>
<tr>
<td>Taiwan*</td>
<td>IAHA</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>IAHA</td>
<td>86</td>
</tr>
<tr>
<td>France*</td>
<td>CEP</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>RIA</td>
<td>21</td>
</tr>
<tr>
<td>Spain*</td>
<td>ID</td>
<td>21</td>
</tr>
<tr>
<td>Senegal*</td>
<td>CEP</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>RIA</td>
<td>117</td>
</tr>
<tr>
<td>Uganda**</td>
<td>CF</td>
<td>90</td>
</tr>
<tr>
<td>Japan**</td>
<td>HA</td>
<td>80</td>
</tr>
<tr>
<td>Uganda**</td>
<td>HA</td>
<td>90</td>
</tr>
</tbody>
</table>

* $P$ = Fisher's exact 2 x 2 test.
PHC = primary hepatic carcinoma, CEP = counter electrophoresis, ID = immunodiffusion, CF = complement fixation, RIA = radioimmunoassay, HA = hemagglutination.

Table 3—Hepatitis B Infection in Primary Hepatic Carcinoma in Mali and Senegal

<table>
<thead>
<tr>
<th>Country</th>
<th>HB$_3$Ag (RIA)</th>
<th>Anti-HB$_3$ (CEP, ID)</th>
<th>Anti-HB$_3$ (HA)</th>
<th>HB$_3$Ag or anti-HB$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>No. positive</td>
<td>Percent positive</td>
<td>No. positive</td>
</tr>
<tr>
<td>Mali</td>
<td>PHC patients</td>
<td>21</td>
<td>10</td>
<td>47.6</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>40</td>
<td>2</td>
<td>5.0</td>
</tr>
<tr>
<td>Senegal</td>
<td>PHC patients</td>
<td>43</td>
<td>29/41</td>
<td>70.7</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>61</td>
<td>6/58</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Adapted from Maupas et al.* and Laouze et al.*
A recessive gene (for review see Blumberg). This simple hypothesis has been formally rejected by the finding that some offspring of matings in which both parents are positive are not carriers. There are, in addition, some other exceptions to simple genetic control. There is a sex effect (in general, males are more often carriers than females) and an age effect (the frequency is low in the very young, increases to a peak in early decades of life, and then decreases in later years). In addition, there is a striking maternal effect and this will be reviewed here.

In completed (or nearly completed) families, if the mother is positive and the father negative, many more of the offspring have HBVAg than in families where the father is a carrier and the mother is not, or when neither parent is a carrier (Table 4). This distribution varies from population to population and in some (for example, in the village of Plati, Greece) this trend is not seen.

In another study on maternal effect, Mazzur et al. used data on the subtypes of HBVAg to investigate its family distribution. They examined families from Bougainville, Papua, New Guinea, in which HBVAg was segregating and where several different subtypes of HBVAg were present. In families in which the mother was positive, essentially all the children had the same subtypes as the parent, but they had variable subtypes when the father was the positive parent or when neither parent was positive. This suggests, as does the previous data, that transmission can occur directly from the mother to the offspring either in utero, early in life, or later during the contact between mother and children.

There is also more direct information on mother–child transmission. Schweitzer and others have shown that when mothers have hepatitis and HBVAg in their blood at the time of delivery, their offspring are very likely to become carriers within weeks or months of birth. With the availability of more sensitive methods for detection of HBVAg, the antigen has now been found in low levels in some cord bloods and newborn children. Nishioka and his colleagues in Japan have shown that mothers who are carriers but do not have apparent hepatitis at the time the child is delivered are also likely to transmit the antigen to their children. This is also true in Taiwan, as Stevens and his colleagues have shown, but it is not necessarily the case in western countries such as United States. In Taiwan, the probability of a child's developing anemia increased in direct proportion to the levels of HBVAg in the mother's blood.

Taken together, it appears that there is a maternal effect. Are there any detectable effects of parental carriage of HBVAg, in addition to the development of carriers among the offspring? An effect, found by Hesser and

### Table 4—Maternal Effect

<table>
<thead>
<tr>
<th>Location</th>
<th>Father positive</th>
<th>No. of families</th>
<th>No. of Children</th>
<th>HBVAg (+)</th>
<th>HBVAg (-)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cebu, Inc.</td>
<td>7</td>
<td>20</td>
<td>15</td>
<td>2</td>
<td>15</td>
<td>0.67 x 10^5</td>
</tr>
<tr>
<td>Bougainville</td>
<td>28</td>
<td>68</td>
<td>8</td>
<td>6</td>
<td>62</td>
<td>0.17 x 10^4</td>
</tr>
<tr>
<td>Turin</td>
<td>7</td>
<td>26</td>
<td>17</td>
<td>1</td>
<td>16</td>
<td>0.09</td>
</tr>
<tr>
<td>Laus, Malta</td>
<td>6</td>
<td>17</td>
<td>17</td>
<td>1</td>
<td>16</td>
<td>0.09</td>
</tr>
<tr>
<td>Beisan, India</td>
<td>6</td>
<td>19</td>
<td>19</td>
<td>1</td>
<td>18</td>
<td>0.09</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>135</td>
<td>135</td>
<td>12</td>
<td>124</td>
<td>0.55 x 10^4</td>
</tr>
</tbody>
</table>

Number of children with HBVAg and without HBVAg in matings in which at least one parent has HBVAg. The matings in which father is HBVAg (+) and mother HBVAg (-), in which both are positive, in every population, but not in Taiwan (45-51, 1972).
her colleagues\textsuperscript{19,20} may, if substantiated by subsequent studies, indicate that the action of the HBV agent does have an effect before the birth of the child. They found that the presence of HBsAg in either parent results in an alteration of sex ratio. In Plati, Greece, the sex ratio ($100 \times$ number of males:number of females) of matings in which either parent has HBsAg (155) is significantly higher than in matings in which neither parent has HBsAg (112) (Table 5). In subsequent studies in four communities in Bougainville and the Solomon Islands, the effect of HBsAg in the parent on the sex ratio of offspring was confirmed.\textsuperscript{20} In this case, however, the direction of the change was different than in Plati. When HBsAg was present in the mother, then there was a decrease in the number of males born; this finding was consistent in each of the communities (Table 6).

An additional effect has been reported by Kukowski et al.\textsuperscript{21} A small but significant increase in gestation time was found in mothers who were HBsAg carriers. If this finding is confirmed, then it would indicate a further profound effect of HBV on the biology of parturition.

**Presence of Hepatitis B Virus in the Mothers of Patients With Hepatoma**

Ohbayashi and his colleagues\textsuperscript{22} reported studies on three Japanese families of patients with hepatoma. In these families, the mothers were HBsAg carriers, and the implication has been drawn that transmission through the mother—as discussed in the previous section—may have an effect on the development of hepatoma in some of the children. We have recently completed controlled studies in Mali and Senegal, west Africa, on patients with hepatoma, their mothers, and other members of their families. These studies will be reported in detail shortly;\textsuperscript{14} preliminary results are given here (Table 7). A large percentage of the mothers of these patients are carriers of HBsAg, supporting the notion that a maternal effect may be significant in the development of hepatoma.

Table 5—Sex Ratio of Offspring from Matings of Parents With and Without HBsAg in a Greek Population

<table>
<thead>
<tr>
<th>No. of Mothers</th>
<th>No. of offspring</th>
<th>Sex ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Mother HBsAg(+)</td>
<td>27</td>
<td>49</td>
</tr>
<tr>
<td>Father HBsAg(+)</td>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>Total, either parent HBsAg(+)</td>
<td>47</td>
<td>85</td>
</tr>
<tr>
<td>Parents HBsAg(-)</td>
<td>172</td>
<td>287</td>
</tr>
</tbody>
</table>

Table 7—HBsAg in Parents of Patients With Primary Hepatic Carcinoma and Parents of Matched Controls*

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>HBsAg(+) (RIA)</th>
<th>Percent HBsAg(+)</th>
<th>No.</th>
<th>HBsAg(+) (RIA)</th>
<th>Percent HBsAg(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHC patients</td>
<td>28</td>
<td>22</td>
<td>78.6</td>
<td>28</td>
<td>16</td>
<td>57.1</td>
</tr>
<tr>
<td>Fathers</td>
<td>27</td>
<td>5</td>
<td>18.5</td>
<td>Fathers</td>
<td>27</td>
<td>5</td>
</tr>
<tr>
<td>Mothers</td>
<td>28</td>
<td>20</td>
<td>71.6</td>
<td>Mothers</td>
<td>28</td>
<td>4</td>
</tr>
</tbody>
</table>

2 x 2 tests:
- PHC patients vs. matched controls 0.15
- Fathers of PHC vs. fathers of controls NS
- Mothers of PHC vs. mothers of controls $3.1 \times 10^{-3}$
- Mothers of PHC vs. fathers of PHC $1.8 \times 10^{-3}$
- Mothers of controls vs. fathers of controls NS

* Adapted from Larouze et al.14
1 Fisher's exact test.

The Development of a Vaccine Against Hepatitis B Virus

Shortly after our discovery of Australia antigen and its association with hepatitis, it became apparent that the antigen found in carriers could be used to prepare a vaccine which might be protective against HBV. We devised a method using ultracentrifugation to separate the antigenic material from possibly infectious particles, and techniques for removing excess serum proteins and insuring that any remaining infectious material would be inactivated.23,24 This was a unique method for producing a vaccine. The antigen is removed from the blood of carriers who have large amounts of the material and given in small quantities to other individuals who require protection.

A series of successful animal trials using chimpanzees and some preliminary human studies have now been completed in several laboratories and they provide encouraging support for the use of the vaccine as a preventative for hepatitis and, probably, for the prevention of the carrier state. Trials of the vaccine in humans are now being considered.25

Consideration of Strategies to Prevent Hepatoma in High-Incidence Areas

If the vaccine is indeed protective, then several strategies for control of hepatoma can be suggested.

There has been considerable discussion on the possibility of developing a vaccine against cancer. A frequent objection to this procedure is that the number of cases of a given cancer (say leukemia) is very small compared to the number of individuals who are theoretically at risk and would
require vaccination. In the case of the hepatitis B vaccine we have described, its use would be justified on the basis that it prevents acute and chronic hepatitis, chronic liver disease, and the development of carriers. If it also prevents hepatoma, then this would be an added important advantage of the vaccination program. In areas with a high endemicity of HBV, hepatitis and chronic liver disease are common in the population, and the use of the vaccine could be medically and economically justified on this basis.

The simplest strategy may be to vaccinate large segments, or conceivably all, of the population in high endemicity areas in Africa, Asia, or elsewhere. If this were the case, then it would first be necessary to know what effect this vaccine would have on carriers of HBsAg, since it is conceivable that it might be disadvantageous. If so, then screening of the population for carriers would be required.

Another strategy might be to specifically treat the newborn offspring of HBsAg-positive mothers. In some parts of the world it has been estimated that most carriers originate from carrier mothers, and the data from Japan and Africa indicate that these children may have a higher risk of developing hepatoma in later life. It has been found that high titer anti-HB immunoglobulin given to newborn offspring of positive mothers can prevent the development of the carrier state in these children. Hence a anti-HB procedure might be developed to provide immunoglobulin shortly after birth and vaccine later in life. Since the endemic areas infection with HBV is likely to be common, recurrent stimulation of the immune system would produce anti-HB.

Any vaccine program should be coordinated with general sanitary and public health measures to eliminate HBV from the general environment, i.e., improvement in waste elimination, control of arthropod vectors, protection of food from contamination, etc.

Discussion

The advances in recent years in our understanding of the biology of Australia antigen and its recognition as the surface antigen of the hepatitis B agent have led to consideration of the feasibility of the immunologic prevention of a common cancer. In considering such an extensive public health program, attention must be given to the possible adverse consequences to the community and population. The removal of an agent such as HBV in highly endemic areas may have consequences which cannot be foreseen. It is for this reason that we are attempting to learn as much as possible about the ecologic interactions of this agent. For ex-
ample, our findings of an association of HBV in parents with the sex ratio of their offspring raise the question whether removal of the agent may affect the population dynamics and sociologic structure of a community. It may be possible to anticipate consequences of this nature in the overall planning of a prevention campaign.

References

1. Beasley RP: Personal communication
16. Hesser JE, Hadziyannis S, Economidou I, Blumberg BS: Family studies of HBsAg in Plati, Greece. (Unpublished data)

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HOST RESPONSES TO HEPATITIS-B
INFECTION IN PATIENTS WITH PRIMARY
HEPATIC CARCINOMA AND THEIR FAMILIES
A Case/Control Study in Senegal, West Africa

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G. Sahmot  B. G. Werner
E. D. Lustbader  M. Fayet
B. S. Blumberg

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Medecine et d'Epidemiologie Africaine, Hopital Claude
Bernard, Paris, France

Summary A case/control study of patients with primary hepatic carcinoma (P.H.C.) and their families was carried out in Dakar, Senegal. 28 P.H.C. cases were matched by age, sex, and ethnic group with 28 controls. Serum was collected from cases, controls, parents (28 mothers, 27 fathers) of cases, parents of controls, 71 siblings of cases, and 58 siblings of controls. Assays of their sera for hepatitis-B surface antigen (HBsAg), antibody to HBsAg (anti-HBs) and antibody to hepatitis-B core antigen (anti-HBc) produced the following results. (1) Nearly all P.H.C. cases (97%) and controls (93%) had some evidence of infection with hepatitis B virus (H.B.V.), but the cases were more likely to be anti-HBc(+) and less likely to be anti-HBs(+) than the controls. (2) Most of the mothers of the cases were HBsAg(+) (71%), whereas only 14% of the mothers of controls were HBsAg(+). Lower titres of anti-HBs were more common in the mothers of the cases. (3) None of 27 fathers of cases had detectable anti-HBs, but 13 (48%) of the fathers of controls were anti-HBs(+). (4) Siblings of the P.H.C. cases were more likely to have anti-HBs than either their sibs with P.H.C. or the sibs of the controls. However, sibs of P.H.C. cases had lower titres of anti-HBs than the sibs of the controls. These data are consistent with the hypothesis that the P.H.C. cases were infected with H.B.V. by their mothers and that there was an environmental factor which affected the immunological response of all family members to H.B.V. Infection with H.B.V. and the mode of response to that infection among members of families appear to be major factors in the etiology of P.H.C. in West Africa.

Introduction

Infection with hepatitis-B virus (H.B.V.) is associated with post-necrotic cirrhosis and primary hepatic carcinoma (P.H.C.). Hepatitis-B surface antigen (HBsAg) is more prevalent in areas where P.H.C. is common, and HBsAg titres are higher in P.H.C. patients than in controls from the same area when assayed by the most sensitive techniques. In Senegal and Mali, we found that 90% and 75%, respectively, of the P.H.C. patients had antibody to hepatitis-B core antigen (anti-HBc) in their blood, and that nearly all the patients had some evidence of hepatitis-B infection (i.e., HBsAg, anti-HBc, and/or anti-HBs). These frequencies are much higher than in matched controls from the same countries (anti-HBc 27.6% in Senegal and 16% in Mali; any evidence of hepatitis-B infection, 65% in Senegal and 61-7% in Mali).

Carriers of HBsAg cluster in families and several workers have demonstrated transmission of H.B.V. from mother to offspring. The risk of individuals infected in utero or shortly after birth of becoming chronic carriers is apparently high.

Several investigators have described a clustering of HBsAg carriers and chronic hepatic disease in families of patients with P.H.C. Ohbayashi et al. reported three such families, each with an HBsAg(+ ) mother, and suggested that maternal transmission of H.B.V. may be an important factor in hepatocarcinogenesis. Beasley tested the mothers of 6 Taiwanese patients with P.H.C.; all were HBsAg(+).

Taken together, these observations suggest that: (1) different modes of transmission of, and different host responses to, hepatitis-B infection cluster in families, and (2) particular kinds of transmission and host response are associated with P.H.C. If this is so, such modes of transmission and types of host response would constitute risk factors for P.H.C. In this study we have tested these hypotheses, identified risk factors, and calculated the relative and absolute risk for P.H.C. contributed by these factors.

Material and Methods

The patients were from the department of medicine of the Le Daniec Hospital, Dakar, Senegal. The diagnosis of P.H.C. was based on the following criteria: (1) palpable mass in the liver, or an enlarged, hard liver; (2) histological examination of a transparietal liver biopsy speci-
<table>
<thead>
<tr>
<th>Evidence</th>
<th>P.M.C. Cases</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>+</td>
<td>%</td>
</tr>
<tr>
<td>HBsAg + I.D.</td>
<td>28</td>
<td>9</td>
<td>32.1%</td>
</tr>
<tr>
<td>HBsAg + R.A.A.</td>
<td>28</td>
<td>22</td>
<td>78.6%</td>
</tr>
<tr>
<td>anti-HBc +</td>
<td>28</td>
<td>23</td>
<td>89.2%</td>
</tr>
<tr>
<td>anti-HBs +</td>
<td>28</td>
<td>7</td>
<td>25.0%</td>
</tr>
<tr>
<td>HBsAg +, anti-HBc (+), and/or anti-HBs (+)</td>
<td>28</td>
<td>27</td>
<td>96.4%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mothers of P.M.C. cases</th>
<th>Mothers of controls</th>
<th>Evidence</th>
<th>No.</th>
<th>+</th>
<th>%</th>
<th>No.</th>
<th>+</th>
<th>%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg + I.D.</td>
<td>28</td>
<td>15</td>
<td>55.6%</td>
<td>28</td>
<td>3</td>
<td>10.7%</td>
<td>1 x 10^{-3}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg + R.A.A.</td>
<td>28</td>
<td>20</td>
<td>71.4%</td>
<td>28</td>
<td>4</td>
<td>14.3%</td>
<td>3 x 10^{-4}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-HBc (+)</td>
<td>28</td>
<td>20</td>
<td>71.4%</td>
<td>28</td>
<td>9</td>
<td>32.1%</td>
<td>6 x 10^{-3}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-HBs (+)</td>
<td>28</td>
<td>3</td>
<td>10.7%</td>
<td>28</td>
<td>15</td>
<td>53.6%</td>
<td>1 x 10^{-3}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg +, anti-HBc (+), and/or anti-HBs (+)</td>
<td>28</td>
<td>21</td>
<td>75.0%</td>
<td>28</td>
<td>19</td>
<td>67.9%</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fathers of P.M.C. cases</th>
<th>Fathers of controls</th>
<th>Evidence</th>
<th>No.</th>
<th>+</th>
<th>%</th>
<th>No.</th>
<th>+</th>
<th>%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg + I.D.</td>
<td>27</td>
<td>2</td>
<td>7.4%</td>
<td>27</td>
<td>3</td>
<td>11.1%</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg + R.A.A.</td>
<td>27</td>
<td>5</td>
<td>18.5%</td>
<td>27</td>
<td>5</td>
<td>18.5%</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-HBc (+)</td>
<td>27</td>
<td>5</td>
<td>18.5%</td>
<td>27</td>
<td>8</td>
<td>29.6%</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-HBs (+)</td>
<td>27</td>
<td>5</td>
<td>0.0%</td>
<td>27</td>
<td>13</td>
<td>48.1%</td>
<td>3 x 10^{-4}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg +, anti-HBc (+), and/or anti-HBs (+)</td>
<td>27</td>
<td>5</td>
<td>18.5%</td>
<td>27</td>
<td>18</td>
<td>66.6%</td>
<td>7 x 10^{-5}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Any evidence of infection with H.A.V.
of the controls and their parents. In the patients p.h.c. h.b.v. However, the some evidence of infection with patients and controls and most of their mothers showed parents is shown in table i. Essentially all the p.h.c. cases and controls and their siblings as possible—71 in the p.h.c. families (40 males, 31 females) and 58 from 22 of the 28 control families (27 males, 31 females).

Blood was collected by venepuncture into dry glass tubes. The sera were separated from the red cells, frozen immediately at -4 °C, and were eventually shipped to Paris and then to Philadelphia where laboratory tests were performed. The sera were kept frozen in dry ice during transportation until the time of testing. All tests were done using coded specimens and the results were recorded without knowledge of the classification of the sera. HBsAg was determined by radioimmunoassay ('Austria' II, Abbott Laboratories) and anti-HBs by hemagglutination, and anti-HBc by counter-immunoelectrophoretic and t.d. techniques. The data were coded and stored in computer disc files from which the tables were computed and analysed by Fisher's exact test, Z score, and the Mann-Whitney test.

**Results**

The frequency and distribution of factors related to H.B.V. infection in the P.H.C. cases and controls and their parents is shown in table i. Essentially all the P.H.C. patients and controls and most of their mothers showed some evidence of infection with H.B.V. However, the nature of the responses of the patients and their parents to H.B.V. infection differed radically from the responses of the controls and their parents. In the P.H.C. patients anti-HBc was more common and anti-HBs less common than in the controls. 20 of 28 mothers of cases were HBsAg(+) and anti-HBc(+), whereas only 4 of the mothers of controls had HBsAg and 9 (including the 4 mothers of cases) had anti-HBc. The remaining 5 anti-HBc(+) control mothers also had anti-HBs. The frequency and titres of anti-HBs were much higher in the control mothers than in the mothers of the P.H.C. patients (the 3 anti-HBs(+) mothers of cases had titres of 1/4, 1/8, and 1/8).

Although frequencies of HBsAg and anti-HBc were similar in the fathers of the cases and the fathers of the controls, none of the 27 fathers of the P.H.C. patients had anti-HBs compared with 13 anti-HBs(+) among 27 fathers of controls.

These data suggest that the mothers of P.H.C. patients are carriers of H.B.V. and the fathers have an inadequate (or different) humoral immunological response to this infectious agent; and further that the mothers transmitted the hepatitis-B infection to their affected offspring. To test the last hypothesis we compared the frequencies of HBsAg in the mothers of anti-HBc(+) cases and anti-HBc(+) controls (table ii). Anti-HBc(+) P.H.C. patients were significantly more likely to have an HBsAg(+) mother (p = 4 x 10^-4) than were anti-HBc(+) controls. In every instance either the P.H.C. patient, his mother, or both showed evidence (HBsAg(+) and/or anti-HBc(+) of current and presumably chronic infection with H.B.V.

A related question is, what is the relative risk of P.H.C. which can be attributed to a mother being a chronic carrier of HBsAg? Since each control was carefully matched with each case, a matched-pair analysis was done. A pair consists of a mother of a case and the mother of the matched control. There were 4 pairs in which both the mother of the case and the mother of the control were HBsAg(+), 16 pairs in which the mother of the case was HBsAg(+) and the mother of the control HBsAg(-), and 8 pairs in which both mothers were HBsAg(-). In no instance was the mother of the control HBsAg(+) and the mother of the case HBsAg(-). Relative risk (R) for matched pairs was calculated by the number of mother pairs in which the mothers of cases were HBsAg(+)/the number of mother pairs in which the mother of the control was HBsAg(+). This yields a ratio of 20/4 or a relative risk of 5/1. An alternative
method of calculating, R, which does not utilise the
matched-pair design but holds constant the other factors
contributing to risk of p.i.c. (absence of anti-HBs in
fathers and absence of anti-HBs in cases or controls),
yields a relative risk of 12.3 if the mother is HBsAg(+).

The other factors which show the most significant dif¬
fERENCE in table 1 give lower relative risks (R) by
matched-pair analysis. For fathers not having anti-HBs,
R=1.95, and for the case being anti-HBs(—), R=2.1.

The power of each of these risk factors to discriminate
between the P.H.C. and control matched pairs is shown
in a branching diagram (see accompanying figure). At
each branch point there was a different distribution of
case control pairs according to the particular risk factor.
In any pair, if the case or the parent of the case was dis¬
tinguished from the control or the parent of the control
by that factor, the branching stopped. For example, in
16 pairs the mother of the case was HBsAg(+) and the
mother of the control was HBsAg(—). For these 16 pairs,
HBsAg(+) was a distinguishing factor. The remaining
11 pairs were then examined to determine whether the
fathers were anti-HBs(+) or (—). In 6 pairs the father
of the case was anti-HBs(—) and the father of the con¬
trol was anti-HBs(+), &c. Ultimately, two pairs
remained that were not distinguished by this procedure.
These 2 controls had all three risk factors and in addition
were HBsAg(+) and anti-HBc(+). From this analy¬
thesis we conclude that these individuals are at high risk of
developing P.H.C.

A matched-pair analysis of the P.H.C. cases and their
parents, and the controls and their parents, for the three
risk factors is shown in table III. If all cases were cor¬
rectly classified by the three factors, they would be
entered above the diagonal. Misclassifications, control
families having more risk factors than cases, would
appear below the diagonal. Cases which cannot be dis¬
tinguished from controls would fall on the diagonal. In
this study there were no misclassifications but there were
5 families of cases which have the same number of risk
factors as their matched control families. We devised a
scoring system (see legend to table III) for evaluating the
significance of this distribution of risk factors and by
this analysis the risk factors were found to be distributed
differently among the case than among the control fami¬
lies.

The analysis of the siblings of the cases and the con¬
trols was complicated by the variable size of the families

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TABLE III—MATCHED-PAIR ANALYSIS OF RISK FACTORS

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<th>No. of risk factors in cases and their parents</th>
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<tr>
<td>Totals</td>
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<td>2</td>
<td>10</td>
<td>15</td>
<td>27</td>
</tr>
</tbody>
</table>

* Risk factors are: mother HBsAg(+), father anti-HBC(-), case or control anti-HBC(-).

Scores—A risk factor which is present in a P.H.C. family adds one point and each risk factor that the matched control family lacks also adds one point to the matched pair. If a case family has 3 risk factors and a control family 0, then the score is 6/6. If a control family has 3 risk factors and a case family 0, then the score is 0/6. For each of the possible combinations in between an appropriate score between 1/6 and 5/6 is given in italics the upper right-hand corner of each box. The best total score if case families all had 3 risk factors and control families 0 is 27. If the scores were randomly distributed the expected score would be 13.5. The observed score was significantly different from 13.5. Total score: 20.16; Z=2.56; p<0.01.

and the failure to collect blood from all siblings. (Sera were not obtained from siblings in 6 of the control families.) With these reservations in mind, certain features are apparent (table IV). Most of the siblings of the P.H.C. patients had been exposed to hepatitis-B infection; 67 of 73 had either HBsAg, anti-HBC, or anti-HBs in their blood. They differed from their affected siblings (the P.H.C. cases) in that their response to hepatitis-B infection was manifested mainly by the production of anti-HBs (82%), and less commonly by the presence of HBsAg or anti-HBC (21%). That is, given parents who confer on their offspring a high risk of developing P.H.C., the ability to produce anti-HBs substantially reduces that risk.

The siblings of the controls had frequencies of HBsAg, anti-HBC, and anti-HBs that were lower than their own brothers or sisters selected as the controls for the P.H.C. cases but were similar to those observed in the general population of Senegal. Comparison of the frequencies of H.B.V.-related antigens and antibodies in the sibs of the cases with those in the sibs of the controls revealed a significantly higher (p=1.6x10^{-5}) frequency of anti-HBs in the sibs of the cases. Since these individu-
ollows were living, or had lived, with a mother and/or a sibling (in whom P.H.C. developed) who were carriers of HBsAg, this is not surprising. However, the titres of anti-HBs/ay and anti-HBs/ad in the sibs of cases were lower than the titres in the sibs of the controls (based on only those individuals who have detectable antibody). The difference was most significant (p=0.001) for antibody against HBsAg/ay, the major antigenic subtype in West Africa.

Discussion

The results of this study are consistent with the hypothesis that a particular mode of transmission—e.g., infection of offspring early in life by mothers who are carriers of H.B.V., is an important (and perhaps necessary) factor in the development of P.H.C. 20 of 28 mothers of P.H.C. patients were HBsAg(+). Since their affected children were born as long as 40 years before this study was done, two interpretations must be considered: (1) the mothers had been infected by the offspring with P.H.C. and HBsAg; and (2) the mothers of P.H.C. patients were chronic carriers of HBsAg (and presumably H.B.V.) and transmitted the H.B.V. infection to the P.H.C. case at birth or early in life.

P.H.C. mothers were probably chronic carriers of HBsAg (and H.B.V.). In point prevalence studies, chronic carriers are much more likely to be detected than are individuals who are transiently HBsAg(+). Furthermore, it is unlikely that these women reached old age in Senegal without having been exposed to H.B.V. infection earlier in life. There was evidence of H.B.V. infection (HBsAg, anti-HBc, or anti-HBs) in about 65% of the population in West Africa, in 60% of women in the reproductive age-group, and in 50% of the 10–15 year age-group (unpublished data). Therefore, we think it is unlikely that the P.H.C. mothers (women now in their 40s, 50s, and 60s) were infected by the offspring with P.H.C. and HBsAg.

If the mothers we detected as being HBsAg(+) were positive at the time their child was born, then they have been HBsAg(+) for 20–40 years. Since chronic carriers are often not carriers forever, it is quite possible that the 8 HBsAg(-) mothers were HBsAg(+) when their affected children were born. (The 8 offspring with P.H.C., of the 8 HBsAg(-) mothers, are currently anti-HBc(+) and 6 of the 8 are HBsAg(+) in their homes and in the community, their immunological response to the agent was inadequate (or, at least, different from other men in Senegal). The nature of the factor in the fathers which affects their immunological response is unknown and will be investigated further. Some insight into the nature of this factor is provided by the observations that the frequencies of anti-HBs are also lower in the cases and the mothers of the cases than in their respective controls, and the titres of anti-HBs/ay are lower in the sibs of the cases than in the sibs of the controls.

These findings suggest that an environmental factor(s), present in the household of the cases, to which the fathers are maximally exposed and to which other family members are exposed to a lesser degree, may be responsible for suppressing the antibody response to HBsAg. Such a factor(s) could be one of the hepatic carcinogens proposed by others (aflatoxins, nitrosamines, cycads).

Irrespective of why the mothers have HBsAg and the fathers lack anti-HBs, the use of these markers as risk factors has identified individuals at high risk of developing a particular cancer. The relative risks we have calculated are conservative estimates. The odds ratio (AD/BC in a 2x2 table), is the usual method for calculating relative risk. Using this approach the relative risk (R) of P.H.C. given by a mother being HBsAg(+) was 15 and for a father lacking anti-HBs, R=59. (Because there are no fathers of cases with anti-HBs (bin B=0), 0.5 was added to each bin to permit estimation of R.) R for absence of anti-HBs in the P.H.C. cases was 5-4. In addition, control subjects in this study had much higher frequencies of HBsAg (57%) and anti-HBc (64%) than we have found in studies of general populations in West Africa or in age, sex, ethnic group matched controls selected for other patients with P.H.C. The controls selected for this study live in the same neighbourhoods as the P.H.C. patients, and they may be at higher risk of developing P.H.C. than controls outside this neighbourhood.
Nevertheless, very few factors associated with common cancers have given anything like the 5 to 12 fold relative risk or the 71-6% absolute risk of H.B.sAg(+) as associated with HBsAg(+) mothers reported here. Anderson\textsuperscript{50} identified a small group of women, with a sister and mother with premenopausal bilateral breast cancer, who have a 47-fold (by odds ratio) relative risk of developing breast cancer, but they account for only 1 or 2% of the breast-cancer population. Smoking cigarettes is associated with a high absolute risk of lung cancer. Yet the relative risk (using similar methods of calculation) for smokers of more than one pack of cigarettes a day is about 10-fold that of non-smokers.\textsuperscript{49}

The results reported here are based on a small sample. The conclusions we have drawn require confirmation, and the hypothesis we have stated with respect to risk factors must be tested. Retrospective and prospective studies have been designed, and the field work is in progress. Should these studies confirm these results it would suggest that prevention or control of infection with H.B.sAg by public-health measures, hepatitis-B hyperimmune globulin, or hepatitis-B vaccine would be followed by a decline in the incidence of P.H.B.s.

We thank Prof. M. Sankal\textsuperscript{6} and Prof. B. Diop of the University of Dakar Medical School and Le Daniec Hospital for their assistance and cooperation with this project.

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REFERENCES

Australia Antigen and the Biology of Hepatitis B

Baruch S. Blumberg

The discovery of the infectious agent associated with hepatitis B and the elucidation of new mechanisms for its dissemination are the consequences of a series of studies involving many investigators in our laboratory in Philadelphia. The particular directions the work has followed have been a product of the interests and personalities of the investigators, physicians, technicians, students, and others who have come to our laboratory. It has resulted in a complex body of data which crosses the boundaries of several disciplines. I have been fortunate in having as co-workers dedicated and highly motivated scientists. We have had a warm, friendly, and congenial atmosphere and I am grateful to my colleagues for bringing these qualities to their work.

Polymorphism and Inherited Variation

E. B. Ford, the Oxford zoologist, lepidopterist, and geneticist, defined polymorphism as "the occurrence together in the same habitat of two or more (inherited) discontinuous forms of a species, in such proportions, that the rarest of them cannot be maintained merely by recurrent mutation" (1). Examples of polymorphism are the red blood cell groups in which the different phenotypes of a system may occur in high frequencies in many populations. This, according to Ford’s view, would be unlikely to occur as a consequence of recurrent mutation operating alone to replace a phenotype lost by selection. Another example is the sickle cell hemoglobin system. In this, \( Hb^s \) genes may be lost from the population each time a homozygote (who has sickle cell disease) fails to contribute to the next generation because of death before the reproductive age. The heterozygotes (\( Hb^s/Hb^a \)) are, however, thought to be differentially maintained in the population because individuals with this phenotype are less likely to succumb to falciparum malaria and consequently survive to contribute genes to the next generation. The theory implies that there are different selective values to the several forms of polymorphisms. This notion has been questioned recently since it has been difficult to demonstrate selective differences for most polymorphisms. Independent of the biological causes for the generation and maintenance of polymorphisms, the concept unifies a large number of interesting biological data. No two people are alike, and polymorphisms probably account for a great deal of variation in humans. There are other interesting implications of polymorphisms. In some instances, the presence of a small amount of a material may be associated with one effect, and the presence of larger amounts of the same material may be associated with a very different effect. One gene for hemoglobin S protects against malaria, while two genes result in the (often) fatal sickle cell disease. Polymorphisms may produce antigenic differences. Antigenic variants of ABO and other red blood cell groups may result in transfusion reactions. Differences in Rh red blood cell groups may cause life-threatening antigenic reactions between a mother and her child late in pregnancy and at the time of birth. Polymorphic antigens may have an effect when one human's tissues interact with those of another in blood transfusion, transplantation, pregnancy, intercourse, and possibly, as we shall see, when human antigens are carried by infectious agents.

Oliver Smithies (who had been a graduate student of A. G. Ogston, my mentor at Oxford) developed the ingenious starch-gel electrophoresis method that allowed the separation of serum proteins on the basis of complex characteristics of their size and shape. With this, he distinguished several electrophoretically different polymorphic serum proteins (haptoglobins, transferrins, and the like). In 1957 and for several years after, in collaboration with Anthony Allison who was then in the Department of Biochemistry in Oxford, we studied these variants in Basque, European, Nigerian, and Alaskan (2) populations and found striking variations in gene frequencies. At the same time, I acquired experience and some skill in mounting field studies. Using this and similar techniques in the following years, I studied inherited variants in other populations and regions. These included red blood cell and serum groups in Spanish Basques, in Alaskan and Canadian Indians, and in Eskimos; B-aminoisobutyric acid excretion in Eskimos, Indians, and Micronesians; protein and red blood cell antigens in Greeks, and various variants in North and South American Indians and in U.S. blacks and whites (1). We identified several new polymorphisms in animals. With Michael Tombs, another of Ogston’s pupils, we discovered a polymorphism of alpha lactalbumin in the “Zebu” cattle of the pastoral Fulani of northern Nigeria (4). Later, Jacob Robbins and I found a polymorphism of the thyroxine binding prealbumin of Macaca mulatta (5). From these studies, and those of other investigators, the richness and variety of biochemical and antigenic variation in serum became strikingly apparent.

In the summer of 1960, Allison came to my laboratory at the National Institutes of Health. We decided to test the hypothesis that patients who received large numbers of transfusions might develop antibodies against one or more of the polymorphic serum proteins (either known or unknown) which they themselves had not inherited, but which the blood donors had. We used the technique of double diffusion in agar gel (as developed by Professor Ouchterlony of Goteborg) to see whether precipitating antibodies had formed in the transfused patients which might react with constituents present in the sera of normal persons.

After testing sera from 13 transfused patients (defined as a person who had received 25 units of blood or more), we found a serum that contained a precipitating antibody (6). It was a very exciting experience to see these precipitin bands and realize that our prediction had been fulfilled. The antibody developed in the blood of a patient (C. de B., male), who had received many transfusions for
the treatment of an obscure anemia. He was extremely cooperative and interested in our research and on several occasions came to Maryland from his home in Wisconsin for medical studies and to donate blood.

During the course of the next few months we found that the antibody in C. de B.’s blood reacted with inherited antigenic specificities on the low density lipoproteins. We termed this the Ag system; it has subsequently been the subject of genetic, clinical, and forensic studies (7).

We continued to search for other precipitating systems in the serums of transfused patients on the principle that this approach had resulted in one significant discovery and that a further search would lead to other interesting findings. During my last year at Bethesda, Harvey Alter, a hematologist, came to work with us. We also had been joined by Sam Wisnich, a former Navy jet fighter and commercial airlines pilot, who, during a slack period in aviation, came to work in our laboratory as a technician.

In 1963, we had been studying the serums of a group of hemophilia patients from Mt. Sinai Hospital in New York City, which had been sent to us by Richard Rosenfield, the director of the blood bank. Antibodies against the Ag proteins were not common in this group of serums, but one day we saw a precipitin band that was unlike any of the Ag precipitins. It had a different configuration, it did not stain readily with Sudan black (suggesting a low lipid content compared to the Ag precipitin), but it did stain red with azocarmine, indicating that protein was a major component. There was a major difference in the distribution of the serums with which the transfused hemophila patient reacted. Most of the antiseraums to Ag reacted with a large number (usually about 50 to 90 percent of the panel serums), but the serum from the hemophila patient reacted with only 1 of 24 serums in the panel, and that specimen was from an Australian aborigine (8, 9). We referred to the reactant as Australian antigen, abbreviated Au. The original Australian serums had been sent to us by Robert Kirk. We subsequently went to Western Australia to collect and test a large number of additional serums.

We then set out to find out why a precipitin band had developed between the serum of a hemophilia patient from New York and that of an aborigine from Australia. At the outset we had no set views on where this path might lead, although our investigation was guided by our prior experience with the Ag polymorphism. In preparing this “history” of the discovery of antigen Au, I constructed an outline, based on a hypothetico-deductive structure, showing the actual events that led to the discovery of the association of Au with hepatitis. From this it is clear that I could not have planned the investigation at its beginning to find the cause of hepatitis B. This experience does not encourage an approach to basic research that is based exclusively on specific-goal-directed programs for the solution of biological problems.

The next step was to collect information on the distribution of Au and antibody to Au in different human populations and disease groups. We had established a collection of serum and plasma samples, later to develop into the blood collection of the Division of Clinical Research of the Institute for Cancer Research, which now numbers more than 200,000 specimens. The antigen was very stable; blood that had been frozen and stored for 10 years or more still gave strong reactions for Au. There were some instances in which blood had been collected from the same individual for six or more successive years. If the serums were positive on one occasion, they were in general positive on subsequent tests; if negative initially, they were consistently negative. Presence or absence of Au appeared, at least in the early experiments, to be an inherent characteristic of an individual.

We were able to use our stored serums for epidemiological surveys and, in a short time, accumulated a considerable amount of information on the worldwide distribution of Au. It was very rare in apparently normal populations of the United States; only 1 of 1000 serums tested was positive. However, it was quite common in some tropical and Asian populations (for example, 6 percent in Filipinos from Cebu, 1 percent in Japanese, and 5 to 15 percent in certain Pacific Ocean populations). We will come back to a consideration of the hypothesis that was generated from this set of epidemiologic observations after consideration of an interesting disease association discovered at about the same time.

Wisnich had been asked to select from our collection the serums of patients who had received transfusions in order to search for more antiseraums to Au. He decided, however, to use them both as potential sources of antibody and also in the panels against which antiseraums to Au were tested. Included among the transfused serums were specimens from patients with leukemia who had received transfusions. A high frequency of Au, rather than antiseraums to Au, was found in this group. We subsequently tested patients with other diseases and found Au only in transfused patients.

On the basis of these observations we made several hypotheses. Although they sound like alternative ones, they in fact are not; and, over the course of subsequent years, in a sense, all of them have been supported and are still being tested.

One hypothesis stated that, although Au may be rare in normal populations, individuals who have Au are more likely to develop leukemia than are individuals who do not have the antigen. That is, there is a common susceptibility factor which makes it more likely for certain people both to have Au and to develop leukemia. We also suggested that Au might be related to the infectious agent (virus) which is said to be the cause of leukemia.

A corollary of the susceptibility hypothesis is that individuals who have a high likelihood of developing leukemia would be more likely to have Au. Down’s syndrome (Mongolism) patients are more likely to develop leukemia than are other children; estimation of the increased risk vary from 20 to 2000 times that of children without Down’s syndrome. I had, in 1964, moved to the Institute for Cancer Research in Philadelphia to start its Division of Clinical Research. While there we tested the serums of Down’s syndrome patients resident in a large institution and found that Au was very common in this group (approximately 30 percent were Au positive); the prediction generated by our hypothesis was fulfilled by these observations, a very encouraging finding (10). The presence of the antigen in people living closer to Philadelphia also made it possible to study persons with Au more readily. Until this time, all the individuals with Au who had been identified either lived in Australia, or some other distant place, or were sick with leukemia.

Down’s syndrome patients were admitted to the Clinical Research Unit (located in our sister institution, Jeanes Hospital) for clinical study. We found again that the presence or absence of Au seemed to be a consistent feature of an individual. If Au was present on initial testing, then it was present on subsequent testing; if absent initially, it was not found later. In early 1966 one of our Down’s syndrome patients, James Bair, who had originally been negative, was found to have Au on a second test. Since this was an aberrant finding we admitted him to the Clinical Research Unit. There was no obvious change in his clinical status. Because he apparently had devel-
oped a "new" protein, and since many proteins are produced in the liver we did a series of "liver chemistry" tests. These showed that between the first testing (negative for Au) and the subsequent testing (positive for Au) this patient (J.B.) had developed a form of chronic anicteric hepatitis.

On 28 June 1966, the day of J.B.'s admission to the Clinical Research Unit, my colleague, Alton Sutnick, wrote the following dramatic note in the patient's chart.

SGOT [serum glutamic oxaloacetic transaminase] slightly elevated! Prothrombin time low! We may have an indication of [the reason for his conversion to Au+] for his conversion to Au+.

His prediction proved correct. The diagnosis of hepatitis was clinically confirmed by liver biopsy on 20 July 1966, and we now began to test the hypothesis that Au was associated with hepatitis (**1**). First, we compared the transaminase (SGPT, serum glutamic pyruvic transaminase) levels in males with Down's syndrome who had Au and those who did not. The SGPT levels were slightly but significantly higher in the Au(+) individuals. Second, we asked clinicians in Pennsylvania to send us blood samples from patients with acute hepatitis. W. Thomas London and others in our laboratory soon found that many hepatitis patients had Au in their blood early in their disease, but the antigen usually disappeared from their blood, after a few days or weeks. Another dramatic incident occurred which added to our urgency in determining the nature of the relation of Au to hepatitis. Barbara Werner (now Dr.) was the first technician in our laboratory in Philadelphia. She had been working on the isolation of Au by extensions of the methods developed by Alter and Blumberg during the earlier work in Bethesda. Early in April of 1967 she noticed that she was not in her usual good state of health. She was well aware of our observations that Au was related to hepatitis and, one evening, tested her own serum for the presence of Au. The following morning a faint but distinct line appeared, the first case of viral hepatitis diagnosed by the Au test. She subsequently developed icteric hepatitis and, fortunately, went on to a complete recovery.

By the end of 1966 we had found that Au was associated with acute viral hepatitis. In our published report (**10**) we said:

Most of the disease associations could be explained by the association of Au(1) with a virus, as suggested in our previous publications. The discovery of the frequent occurrence of Au(1) in patients with virus hepatitis raises the possibility that the agent present in some cases of this disease may be Australia antigen or be responsible for its presence. The presence of Australia antigen in the thalassemia and hemophilia patients could be due to virus introduced by transfusions.

That is, we made the hypothesis that Au was (or was closely related to) the etiologic agent of "viral" hepatitis, and we immediately set about to test it. Our original publication did not elicit wide acceptance; there had been many previous reports of the identification of the causative agent of hepatitis and our claims were naturally greeted with caution. Indeed, an additional paper on Australia antigen and acute viral hepatitis (**11**) which extended our findings published in 1967 was initially rejected for publication on the grounds that we were proposing another "candidate virus" and there were already many of these.

Confirmation of our findings and the first definitive evidence on the relation of Au to posttransfusion hepatitis came soon. Kazuo Okochi, then at the University of Tokyo, had followed a line of inquiry very similar to ours. He had started with the investigation of antisera to Ag (lipoprotein), and we had corresponded on this subject. Okochi then found an antiserum in a patient with chronic myelogenous leukemia which was different from the precipitins in antiserum to Ag. He also found that it was associated with liver damage. During my several field trips to Japan, I had lectured on Australia antigen. Okochi sent the unusual antiserum to us to compare with antiserum to Australia antigen; we found that they were identical. He confirmed our finding of the association of Au with hepatitis and then proceeded to do the first definitive study of transfusion. He found that Au could be transmitted by transfusion and that it led to the development of hepatitis in some of the people who received it, and that some transfused patients developed antibody to Au (**12, 13**). The Au-hepatitis association was also confirmed in 1968 by Alberto Vierucci (**14**) who worked in our laboratory and Alfred Prince (**15**).

We had made some preliminary observations in Philadelphia in collaboration with John Senior of the University of Pennsylvania on the transfusion of donor blood which was found to contain Au. We then developed a protocol for a controlled, long-term study to determine whether donor bloods which had Au were more likely to transmit hepatitis than those which did not. In 1969 we heard from Okochi that he had already embarked on similar transfusion studies. In June of that year he visited our laboratory in Philadelphia and showed us his data. These, in his (and our) opinion, demonstrated with a high probability that donor blood containing Australia antigen was much more likely to transmit hepatitis than donor blood which did not contain the antigen. [Similar studies were later done by Dr. David Gocke (**16**) in the United States and the same conclusions were reached.] We immediately stopped the experimental study and established the practice of excluding donor bloods with Australia antigens in the hospitals where we were testing donor units. This was a dramatic example of how technical information may completely change an ethical problem. Before Okochi's data had become available it was a moral necessity to determine the consequences of transfusing blood containing Australia antigen; and it had to be done in a controlled and convincing manner since major changes in blood transfusion practice were consequent on the findings. As soon as the conclusion of Okochi's well-controlled studies were known to us, it became untenable to administer donor blood containing Australia antigen. Autres temps, autres moeurs.

It was, however, possible to do a study to evaluate the efficacy of Au screening on posttransfusion hepatitis with the use of historical controls. Senior and his colleagues had completed an analysis of posttransfusion hepatitis in Philadelphia General Hospital before the advent of screening and found an 18 percent frequency of posttransfusion hepatitis. In the fall of 1969, we started testing all donor blood and excluding Au positive donors. Senior and others undertook a similar follow-up study 1 year after the screening program was in progress. They found that the frequency of posttransfusion hepatitis had been reduced to 6 percent, a striking improvement (**17**).

The practical application of our initial esoteric finding had come about only 2 years after the publication of our paper on the association between Au and hepatitis (**10**). In retrospect, one of the major factors contributing to the rapid application of the findings was the simplicity of the immunodiffusion test. Another was our program of distributing reagents containing antigen and antibody to all investigators who requested them. We did this until this function was assumed by the National Institutes of Health.

After the confirmation of the association of hepatitis with Australia antigen, a large number of studies were published, and, in a relatively short time the routine use of the test in blood banks became essentially universal in the United States and many other countries. It has been es-
Virology

Virological methods (that is, tissue culture, animal inoculation, and others) had been used for many years prior to our work to search for hepatitis virus, but had not been very productive. Our initial discoveries were based primarily on epidemiologic, clinical, and serologic observations. Here, I will try to review the early virology work from our laboratory [Robinson and Lutwick have reviewed much of the recent work (18)].

Bayer et al. (19), using the isolation techniques initially introduced by Alter and Blumberg (20), examined isolated Au with the electron microscope. They found particles about 20 nanometers in diameter which were aggregated by antisera to Au. There were also sausage-like particles of the same diameter, but much elongated (Figs. 1 and 2). Subsequently Dane, Cameron, and Briggs identified a larger particle about 42 nm in diameter with an electron-opaque core of about 27 nm (21). It is probable that this represents the whole virus particle. Both the 20-nm and 42-nm particles contain Australia antigen on their surfaces and this is now termed hepatitis B surface antigen (HBsAg). The surface antigen can be removed from Dane particles by the action of detergents to reveal the core which has its own antigen, hepatitis B core antigen (HBcAg). Antibodies to both these antigens (anti-HBs, anti-HBc) can be detected in human blood. The surface antigen can be detected in the peripheral blood by the methods we initially introduced and by more sensitive methods that have since been developed. Anti-HBs is often found in the peripheral blood after infection and may persist for many years. It may also be detected in people who have not had clinical hepatitis. Anti-HBc is usually associated with the carrier state (that is, persistent HBsAg in the blood) but may occur without it. HBcAg itself has not been identified in the peripheral blood. Anti-HBC is also found commonly during the active phase of acute hepatitis, before the development of anti-HBs but in general does not persist as long as anti-HBs.

DNA has been isolated from the cores of Dane particles and is associated with a specific DNA polymerase. Robinson and Lutwick have shown that the DNA is in the form of double-stranded rings (18). Jesse Summers, Anna O’Connell, and Ir¬

Vaccine Against Hepatitis B

In 1968 we were informed by the federal government, which provided most of the funds for our work, that they would like to see applications of the basic research they had funded for many years. It occurred to us that the existence of the carrier state provided an unusual method for the production of a vaccine. We presumed that the very large amounts of HBsAg present in the blood could be separated from any infectious particles and used as an antigen for eliciting the production of antibodies. The antibodies in turn would protect against infection with the virus. Irving Millman and I applied separation techniques for isolating and purifying the surface antigen and proposed using this material as a vaccine. To our knowledge, this was a unique approach to the production of a vaccine; that is, obtaining the immunizing antigen directly from the blood of human carriers of the virus. In October 1969, acting on behalf of the Institute for Cancer Research, we filed an application for a patent for the production of a vaccine. This patent was subsequently (January 1972) granted in the United States and other countries (22).

There are observations in nature which indicate that antibody against the surface antigen is protective. In their early studies, Okochi and Murakami observed that transfused patients with antibody were much less likely to develop hepatitis than those without it (13). In a long-term study, London et al. (24) has shown that patients on a renal dialysis unit, and the staff who served them, were much less likely to develop hepatitis if they had antibody than if they did not (Fig. 4). Lustbader has used these data to develop a statistical method for rapidly evaluating the vaccine (25).

There have now been several animal and human studies of the vaccine, and the results are promising (26). It should be possible to determine the value of the vaccine within the next few years.

Variation in Response to Infection with Hepatitis B

A physician is primarily interested in how a virus interacts with humans to cause disease. But this is only part of the world of the virus. Our introduction to studies on hepatitis B was not through patients with the disease, but rather through asymptomatic carriers and infected individuals who developed antibody. Therefore, many of our investigations have been of infected but apparent-
ly healthy people. There are a variety of responses to infection:

1) Development of acute hepatitis proceeding to complete recovery. Transient appearance of HBsAg and anti-HBc. Subsequent appearance of anti-HBs which may be persistent.

2) Development of acute hepatitis proceeding to chronic hepatitis. HBsAg and associated anti-HBc are usually persistent.

3) Chronic hepatitis with symptoms and findings of chronic liver disease not preceded by an episode of acute hepatitis. HBsAg and anti-HBc are persistent.

4) Carrier state. Persistent HBsAg and anti-HBc. Carrier is asymptomatic but may have slight biochemical abnormalities of the liver.

5) Development of persistent anti-HBs without detectable HBsAg or symptoms.

6) Persistent HBsAg in patients with an underlying disease often associated with immune abnormalities, that is, Down's syndrome, lepromatous leprosy, chronic renal disease, leukemia, primary hepatic carcinoma. Usually associated with anicteric hepatitis.

7) Formation of complexes of antigen and antibody. These may be associated with certain "immune" diseases such as periarteritis nodosa.

Family Studies

In our first major paper on Australia antigen (9) we described family clustering of Au in a Samaritan family from Israel that had been studied by the anthropologist Batsheva Bonne. From it we inferred the hypothesis that the persistent presence of Au was inherited as a simple autosomal recessive trait. The genetic hypothesis has proved to be very useful not in the sense that it is necessarily "true" [exceptions to the simple hypothesis were noted by us and others very soon (27)], but because it has generated many interesting studies on the family distributions of responses to infection with hepatitis B. We suggested that hepatitis virus may have several modes of transmission. It can be transmitted horizontally from person to person similar to the transmission of "conventional" infectious agents. This is seen in the transmission of hepatitis B virus (HBV) by transfusion. Other forms of direct and indirect horizontal transmission exist; for example, by sputum, by the fecal-oral route, and, perhaps, by hematophagous insects (see below). It has even been reported that it has been spread by computer cards (28), an extraordinary example of adaptation by this ingenious agent! HBV may also be transmitted vertically. If the genetic hypothesis were sustained, then it would imply that the capacity to become persistently infected is controlled (at least in part) as a Mendelian trait. The data are also consistent with the notion that the agent could be transmitted with the genetic material; that the virus could enter the nucleus of its host and in subsequent generations act as a Mendelian trait. The data also suggest a maternal effect. A reanalysis of our family data showed that in many populations more of the offspring were persistent carriers when the mother was a carrier than when the father was a carrier. Many investigators have now shown that women who have acute type B hepatitis just before or during delivery or women who are carriers can transmit HBV to their offspring, who then also become carriers. This may be a major method for the development of carriers in some regions, for example, Japan. Interestingly, this mechanism does not appear to operate in all populations. This suggests that some aspects of delivery and parent-child interaction, differing in different cultures, as well as biological characteristics may affect transmission. The family is an essential human social unit. It is also of major importance in the dissemination of disease. A large part of our current work is directed to an understanding of how the social and genetic relations within a family affect the spread of hepatitis virus.

Host Responses to Human Antigens and HBV: Kidney Transplantations

London, Jean Drew, Edward Lustbader, and others in our laboratory have undertaken an extensive study of the patients in a large renal dialysis unit in Philadelphia (24, 29, 30). The renal patients can be characterized on the basis of their responses to infection with hepatitis B. Patients who develop antibody to HBsAg are significantly more likely to reject transplanted kidneys that are not completely matched for HLA antigens than patients who become carriers of HBsAg (Fig. 5) (30). Since many of the patients became exposed to hepatitis B while on renal dialysis, their response to infection can be determined prior to transplantation. In this patient population there is a significant correlation between development of anti-HBs and the subsequent development of antibodies to HLA after transplantation. We have also found a correlation between the development of antibody to HLA and anti-HBs in transplanted hemophila patients and in pregnant women. Hence, there appears to be a correlation between the response to infection with HBV and the immunologic response to polymorphic human antigens in tissue transplants. Further, from preliminary studies, it appears that donor kidneys from males are much more likely to be rejected by patients with anti-HBs than by patients without anti-HBs. These differences were not observed when the kidneys were from female donors. London is now extending his observations to other transplants, in particular, bone marrow, to determine whether a similar relation exists.

Sex of Offspring and Fertility of Infected Parents

In many areas of the world, including many tropical regions (for example, the Mediterranean, Africa, southeast Asia, and Oceania) the frequency of HBsAg carriers is very high. In these regions,
most of the inhabitants will eventually become infected with HBV and respond in one of the several ways already described. Our family studies and the mother-child studies show that there is a maternal effect. Jana Hesser (then a graduate student in anthropology working in our laboratory) and Ioanna Economomidou, Stephanos Hadziyannis, and our other Greek colleagues collected information on the sex of the offspring of parents in a Greek town in southern Macedonia. In this community the probability of infection with HBV is very high and a majority of the parents had evidence of infection, that is, detectable HBsAg or anti-HBs (or both) in their blood. It was found that if either parent was a carrier of HBsAg there were significantly more male offspring than in other matings (31). Using the Greek data and additional data from Mali in West Africa in subsequent studies, London, Drew, and Veronique Barrois (a postdoctoral trainee from Paris) have found that there is a deficiency of male offspring when parents have anti-HBs and that this may be a consequence of differential male mortality during the period in utero (32). This had led London and his colleagues to test the hypothesis that anti-HBs has specificities in common with H-Y or other histocompatibility antigens determined by genes on the Y chromosome. If these observations are supported by additional studies, then HBV may have a significant effect on the composition of populations in places where it is common, which includes the most populous regions of the world. The ratio of males to females in a population has a profound effect on population size as well as on the sociology of the population. This connection of anti-HBs with sex selection may also explain why there is a greater likelihood of rejection of male kidneys by renal patients with anti-HBs, and indicate how kidneys can be better selected for transplantation. Pregnancy and transplantation of organs have certain immunologic features in common. Rejection of male kidneys and "rejection" of the male fetus may be mediated by similar biological effects.

**Table 1. Frequency of HBsAg, anti-HBc, and anti-HBs in primary hepatic carcinoma (PHC) and controls in Senegal and in Mali, West Africa. Abbreviations: RIA, radioimmunoassay; P is the two-tailed probability obtained from Fisher's Exact Test. (Adapted from Larouze et al. (27))**

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**Primary Hepatic Carcinoma**

The project with which we are most concerned at present is (i) the relation of hepatitis B to primary hepatic carcinoma (PHC), and (ii) methods for the prevention of the disease. PHC is the most common cancer in men in many parts of Africa and Asia. For many years investigators in Africa including Payet et al. (33), Davies (34), and Steiner et al. (35) have suggested that hepatitis could be the cause of PHC. With the availability of sensitive tests for Australia antigen it became possible to test this hypothesis; it has now been established that there is a striking association of hepatitis B with PHC (36, 37) (Table 1). In our studies in Senegal and Mali we found that essentially all the patients had been infected with HBV and that most had evidence of current infection (presence of HBsAg or anti-HBc, or both). Ohbayashi and his colleagues (38) had reported several families of patients with PHC in which the mothers were carriers. In our study in Senegal (39), Bernard Larouze and others found that a significantly larger number of mothers of PHC patients were carriers of HBsAg compared with controls, and that none of the fathers of the cases had anti-HBs. In control families, on the other hand, 48 percent of the fathers developed antibody (Table 2). The hypothesis we have made is that, in some families, children will be infected by their mothers, either in utero, at the time of birth, or shortly afterward during the period when there is intimate contact between mother and children. In some cases, the infected child will proceed through several stages to the development of PHC. At each stage, only a fraction of the infected individuals will proceed to the next stage, and this will depend on other factors in the host and
in the environment. The stages include retention of the antigen (carrier state), development of chronic hepatitis, development of cirrhosis and finally, development of PHC (Fig. 6). We are currently testing this hypothesis in prospective studies in West Africa (37). If it is true, then prevention of PHC could be achieved by preventing infection with HBV, and the vaccine we have introduced, in association with appropriate public health measures, could reduce the amount of infection. This might also involve the use of γ-globulin in the newborn children of carrier mothers, and such studies are now being conducted by Beasley and his colleagues in Taipei. We are now considering the appropriate strategies that might be used to control hepatitis infection and, perhaps, cancer of the liver.

Transmission by Insects

HBsAg has been detected by several investigators including Prince et al. (40), Smith et al. (41), Muniz and Micks (42), and others in mosquitoes collected in the field in areas where HBsAg is common in the human population. In 1971, we collected mosquitoes in Uganda and Ethiopia and found Au antigen in individual mosquitoes (43). In more extensive studies in Senegal, we found a field infection rate of about 1 in 100 for *Anopheles gambiae* and also identified the antigen in several other species of mosquitoes (44). It is not known whether HBV replicates in mosquitoes, but it has been reported that it can be detected in mosquitoes many weeks after feeding and it has been found in a mosquito egg. Feeding experiments have been conducted with the North American bedbug (*Cimex lectularius*); these studies show that this insect can also carry the antigen (45). William Wills, in our laboratory, has found a very high infection rate (~60 percent) in the tropical bedbug *Cimex hemipterus* collected from the beds of individuals known to be carriers of hepatitis B (46). Bedbugs could transfer blood (and the virus) from one occupant of a bed to another. If it is in fact a vector of hepatitis, then it could provide a frequent nonvenereal (and unromantic) form of communial spread. It may also provide a means for transmission from mother (or father) to young children who may share the parents’ bed in early life; and this would be related to the child-rearing practices of a community.

Insect transmission may be important in the program for the control of hepatitis B infection and for the prevention of chronic liver disease and primary hepatic carcinoma. An understanding of the role of insects in the spread of infection, particularly its transmission from mother to children, would help in designing effective strategies for control.

Hepatitis B as a Polymorphism

The original discovery of hepatitis B resulted from the study of serum antigen polymorphisms. Its identification as an infectious agent does not diminish the value of this concept. It is useful to view infection with HBV not only as a “conventional” infection but also as a trans fusion or transplantation reaction; our studies on renal transplantation are an example of this.

HBV appears to have only a small amount of nucleic acid, probably only sufficient to code for a few proteins. Much of the coat (and possibly other portions of the virus) could be produced by the genes of the host. Millman and his colleagues found that the surface antigen contains material with antigenic specificities in common with serum proteins including IgG, transferrin, albumin, beta-lipoprotein, and others (47). If this is true, then the antigenic makeup of the virus would be, at least in part, a consequence of the antigenic characteristics of the host from whence it came; and this, as suggested by our sex studies, may include male antigens. In our discussion of the “Icron” concept (a name we introduced which is an acronym on the Institute for Cancer Research) (48), we pointed out that the responses of the putative host to HBV may be dictated in part by the nature of the “match” between the antigens of the host and virus (that is, the virus acts as if it were a polymorphic human antigen). London et al. (49) and Werner and London (50) have described this in a review of these concepts. If person A is infected with HBV particles that contain proteins antigenically very similar to his own, then he will have little immunologic response and will tend to develop a persistent infection with the virus. On the other hand, if the proteins of the agent are antigenically different from his, he will develop an immune response to the virus (that is, anti-HBs) and will have a transient infection. During the course of infection in person A, new particles will be synthesized.

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which contain antigenic characteristics of A. In turn, person A can infect person B and the same alternatives present themselves. If the relevant proteins of B are antigenically similar to the antigens of A and the antigens of the HBV produced by A, then B could develop a persistent infection. If they are different, then antibody can form, as described above. (A derivative of this hypothesis is that inflammatory disease of the liver is associated with the immune response to the infectious agent rather than solely with replication of the agent.) A further possibility is that the virus has complex antigens; that some may match the host and some may not and that both persistent infection and development of antibodies may occur. The persistent antigens and the antibody in the same individual would have different specificities, and this occurrence has been described (51).

This view of the agent as an 1cron introduces an interesting element into the epidemiology of infectious agents in which not only the host and virus are factors, but also the previous host or hosts of the agent. If, in fact, the agent does replicate in insects (see above), the antigenic characteristics of previous human hosts may be affected by transmission through another species. This in turn might have an effect on the response of the next host.

Bioethics and the Carrier State

During the course of our work a number of bioethical questions have arisen (52). Experience has shown that these bioethical considerations cannot be separated from "science," that answers cannot be provided on a "purely scientific" ground, and that our technical knowledge is inseparably intertwined with bioethical concerns.

It has been recognized that hepatitis B may be transmitted by means other than transfusion, that is, by contact, fecal-oral spread, insects, and the like. With the introduction of the screening test, many carriers were identified. It is estimated that there are 1 million such carriers in the United States and more than 100 million in the world. This has led to a situation that may be unique in medicine. Although some carriers may be able to transmit hepatitis by means other than blood transfusion, this is probably not true for many (or most) carriers. There are studies which show that spread of infection from carriers in health care occupations to patients may not be common. At present there is no satisfactory method of identifying the infectious carriers although it appears that carriers with "e" antigen, an unusual antigen originally described by Magnus and shown to be more likely to transmit disease. Despite this, carriers have had professional and social difficulties. Health care personnel who are carriers have been told that they must leave their jobs. In some cases, carriers have changed their pattern of social behavior because of the fear that they might spread disease to people with whom they come in contact. What appeared to be happening was the development of a class of individuals stigmatized by the knowledge that some member of the "class" could transmit hepatitis.

The bioethical problems raised from the studies of hepatitis carriers can be viewed as a conflict between public health interests and individual liberty. When the risk to the public is clear, and the restrictions on personal liberties are small, there is little problem in arriving at appropriate regulations. For example, the transfusion of blood containing hepatitis B antigen is a disadvantage to the patient recipient and it has been stopped. The denial of the right to donate blood is not a great infringement of personal activity, and the individuals concerned and society have agreed to accept this moderate restriction. The problems raised by person-to-person transmission are more difficult. The extent of the hazard to the public is not clear, since it is not known whether all carriers who transmit disease from those who do not. On the other hand, if all carriers are treated as infectious, the hazards imposed on the carrier may be enormous, that is, loss of job and ability to continue in the same profession, restriction of social and family contacts, and others. What is clear is that for a very large number of carriers, the risk of transmitting hepatitis by person-to-person contact must be very small. All members of the carrier class should not be stigmatized because some can transmit hepatitis.

On a broader level, the ethical issue is raised as to the extent to which biological knowledge about individuals should impinge on daily lives. Is it appropriate to regulate the risks inherent in people living together and interacting with each other? An issue has been raised with respect to hepatitis because the test can be easily done and because millions of people are tested as part of blood donor programs. As a consequence of these tests, this particular group of carriers has been identified. There are carriers of other agents, some of them potentially more hazardous (such as staphylococcus or typhoid), and these carriers are not routinely tested and therefore not placed at a disadvantage.

It is hoped that many of these problems can be resolved by continued research into the nature of the hepatitis carrier state, and that carriers who have already been identified will not be jeopardized during this period when necessary information is not available.

A characteristic of many large-scale public health control programs is the emergence of problems that were not anticipated prior to the institution of the program. For example, the control of malaria has in many areas resulted in a markedly decreased infant mortality with a large increase in population. When this has not been accompanied by a concomitant increase in food production, the nourishment and well-being of the population have actually decreased.

With the availability of the serologic and environmental tests for hepatitis B, it is now possible to begin the design of control measures for this disease. If the hepatitis B vaccine is found to be effective, then it may also be of value in preventing the development of the carrier state. We are now attempting to investigate the biology of the hepatitis B agent to learn whether some of the consequences of control can be known before the program begins. An example already discussed is the possible effect of HBV infection on sex ratio. The role of the virus in the life of the insects in which it is found is not known, but may be profound; and there may be other effects on the ecology that are not now obvious.

We hope to continue the study of these broad problems to be as well prepared as possible when and if attempts are made to eliminate or decrease the frequency of the hepatitis B virus.
Hepatitis B Virus and Primary Hepatocellular Carcinoma: Relationship of "Icrons" to Cancer

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Since the identification of the hepatitis B virus (HBV) in 1967, it has become possible to determine the relationship between HBV and primary hepatocellular carcinoma (PHC) (Blumberg et al. 1975). A substantial body of evidence has accumulated supporting the hypothesis that persistent infection with HBV is required for the development of PHC; this evidence is summarized in this paper. It follows that this common and very deadly cancer can be prevented if persistent infection with HBV can be prevented. A vaccine against HBV (Millman and Blumberg 1978) is currently being tested, which, if proved safe and effective, could help in the prevention program. The increasing knowledge of how HBV is transmitted will also help in the design of public-health strategies.

HBV has many unusual characteristics, which are described later in this paper. In 1971 (Blumberg et al. 1971), on the basis of the characteristics then known, we suggested that HBV might be considered, for heuristic purposes, the prototype of a new class of viruses and that others of this new class might be identified in the future. The term "Icron," an acronym for The Institute for Cancer Research, with a neuter Greek ending, was used to designate this class of infectious agents. The relationship of HBV to PHC described in this paper indicates that other members of the postulated Icron class of viruses may also be related to common cancers and, again by analogy to the HBV-PHC model, may be preventable. The woodchuck hepatitis virus (WHV) (Summers et al. 1978a; Werner et al. 1979), described elsewhere in this volume, is very similar to HBV and is also related to cancer of the liver. It appears to be a second example of an Icron. There is good reason to believe that other such viruses will be found, and some of these may be related to other common cancers in man. This study on HBV may then provide a useful general approach to an understanding of the role of viruses in the pathogenesis of cancer and in the prevention of such cancers.

Primary Cancer of the Liver
Primary (in contrast with secondary or metastatic) cancer of the liver originates in the liver and is a malignant tumor of hepatic tissue (parenchymal) cells; hence,
the technical name primary hepatocellular carcinoma. The cancer usually arises in a liver that has already been damaged by chronic inflammation (chronic active hepatitis) and/or scarring (cirrhosis). Because the liver is a large internal organ (approximately 2500 g), the tumor has usually grown to substantial size by the time it is detected by a physician. Surgery, X ray, and drug therapy are rarely effective, and patients with the tumor generally die within 1 year of diagnosis (Okuda and Peters 1976).

It has been recognized for many years that there is a marked variation in the incidence of PHC by geography, race, age, and sex. The tumor is relatively rare in the United States (approximately 6000 cases per year) and in Europe, but it is the most common cancer of males in parts of China, Taiwan, other regions of Southeast Asia, and most of Africa. Annual mortality rates from 25 to 150 per 100,000 have been recorded in some of these areas. In Taiwan, with a population of approximately 17,000,000, about 10,000 persons die annually of PHC. If mortality from PHC is similar in the People's Republic of China and in the other areas of the world where the disease is endemic, then this cancer may cause between 500,000 and 1,000,000 deaths annually. Therefore, it must be one of the two or three most common and most deadly cancers in the world.

In the United States, PHC is more common among nonwhites, with particularly high rates among Americans of Chinese descent. It is also more common among blacks than whites, but not nearly as frequent as among blacks in Africa (Waterhouse et al. 1976).

Among all geographic and racial groups, males are more commonly affected than females. Depending on the age and population reported, male-to-female ratios have varied from 3:1 to 9:1. In studies with our colleagues Chung Yong Kim and Hie-Won Hann in Korea and Bernard Larouzé, Marc Sankale, and Birame Diop in Senegal, we have found five to six times as many cases among men than among women. PHC is rare in children, but we have seen cases in teenagers and in persons in the 20-year-old age group. The highest incidences appear to occur in the 40-60-year-old age group.

An interesting characteristic of primary liver cancer was discovered in 1965 by the Soviet scientists G. I. Abelev and Y. S. Tatarinov (N. F. Gamaleya Institute, Moscow). Abelev found that experimentally induced liver cancers in mice produced a protein that migrated as an α-globulin on electrophoresis and was identical with a protein produced during embryonic life by the yolk sac and the fetal liver. Because of these characteristics, the protein was called α-fetoprotein (AFP). About the same time, Tatarinov described an α-globulin in the sera of humans with PHC. Shortly thereafter, Abelev demonstrated that the human protein was also a fetal protein and was very similar to the mouse AFP (for review, see Abelev 1968). Since then, studies of AFP in the sera of patients with PHC have shown prevalences varying from about 50% in the United States and western Europe to nearly 90% in Africa and Asia (Masseyeff 1973). Because AFP is normally synthesized in only minute quantities after birth, the detection of AFP is useful as a diagnostic test for PHC and has been used to screen nearly a million individuals in the People's Republic of China (Sun, this volume).

In this paper we will not review the extensive work on the induction of liver cancer by chemicals in experimental animals. Many chemicals, including azo dyes, nitrosamines, polycyclic hydrocarbons, cyesine, and mycotoxins, are liver carcinogens in experimental animals. In man, interest has centered on aflatoxin B, a mycotoxin produced by a family of fungi that commonly contaminates peanuts, grains, and other human foodstuffs and has caused
"epidemics" of cancer in lower animals. Aflatoxin has been detected in the environment in many areas of the world that have high frequencies of PHC. It has been very difficult, however, to design and carry out meaningful studies in human populations on the direct relationship of PHC to aflatoxin (i.e., whether, within an area having a high incidence of primary liver cancer, patients with PHC are more likely to have ingested aflatoxin than individuals without the disease). From the point of view of prevention, elimination of aflatoxin from the environment would require major changes in the economic and agricultural practices of the populations involved.

In this paper, evidence is adduced to test the hypothesis that persistent infection with HBV is necessary for the development of cancer of the liver. In testing the hypothesis, the process of independent evidence will be used. Several independent bodies of evidence are considered, any one of which taken by itself may not be convincing, but taken together they provide, we believe, substantial support for this hypothesis.

It is also clear from the evidence presented that although chronic HBV may be necessary for the development of PHC, it is not sufficient, and other factors in the host and environment are required to bring about the pathogenesis, i.e., there are several necessary "causes" of PHC, and HBV infection is one of them. It is a hopeful characteristic of preventive medicine that it is not necessary to know all causes in order to effect prevention; it is necessary to know only one preventable cause to interrupt the cycle. An example often cited is the control of an epidemic of cholera in London in 1848 described by John Snow. His studies were done before the discovery of the cholera vibrio and, in fact, before the promulgation of the germ theory of disease. He found in one neighborhood of London that those persons who drank from a particular common water source, a well, had a much higher frequency of cholera. From this he deduced that drinking water from the well was a preventable cause; he removed the well's handle and thus arrested the epidemic even though he was not aware of the "causative" organism.

Hepatitis B Virus
The virion (infectious particle) of HBV is 42 nm in diameter; it has a coat protein, hepatitis-B surface antigen (HBsAg), and a core protein, hepatitis-B core antigen (HBeAg). The core contains a unique double-stranded circular DNA molecule with a single-strand region and an enzyme, a specific DNA polymerase, that can copy the single-strand region to complete the double-stranded molecule. Recently, another antigen, e antigen (HBeAg), which is soluble and may appear in the serum, has also been associated with the core of the 42-nm particle. Three kinds of virus-associated particles are seen by electron microscopy in the sera of infected individuals—42-nm particles (often called Dane particles after their discoverer), 20-22-nm particles, and rod-shaped particles varying in length but 20-22 nm in width. The latter two particles are present in much greater numbers than the 42-nm virions and are composed entirely of coat protein, HBsAg (for review, see Melnick et al. 1977).

When an individual is infected with the virus, there is an incubation period ranging from 6 days to 6 months before HBsAg appears in the blood. In acute, self-limited infection, HBsAg is present in the circulation for days or weeks, but it eventually disappears and antibody to HBsAg (anti-HBs) becomes detectable. Antibody to the core of the virus (anti-HBc) generally appears before anti-HBs,
when HBsAg is present in the blood, and persists for variable periods after the disappearance of HBsAg. It may be found when neither HBsAg nor anti-HBs is detected and may be associated with active viral replication.

There is a second, qualitatively distinct, host response to HBV. Some individuals when infected with the virus fail to clear it from their blood. They remain HBsAg(+) and anti-HBc(+) for years and produce little or no detectable anti-HBs. These individuals are the "chronic carriers" of HBV. They can, if intact virions are present in their plasma (as indicated by the presence of HBeAg, viral DNA, HBV DNA polymerase, and/or Dane particles), transmit HBV to others via blood transfusion or close personal contact; they are at increased risk of developing several chronic liver diseases, including primary liver cancer.

**HBV and the Development of PHC**

There are nine independent lines of evidence supporting the hypothesis that persistent infection with HBV is necessary for the development of PHC.

1. If two maps of the world are overlaid one on the other, one showing the distribution of the high-incidence (endemic) areas of PHC and the other showing the areas with high frequencies of chronic carriers of HBV in the general population, there is a very high degree of overlap. Thus, PHC occurs commonly in the same regions where chronic carriers are prevalent.

2. The traditional epidemiological method for investigating whether some factor is associated with a disease is the case-control study. In this study, identified cases of disease are compared with appropriate controls without disease (usually matched by age, sex, and location) for the presence or absence of the suspected factor. Such studies and related ones have shown that up to 80% of patients with PHC living in endemic areas are HBsAg(+) and up to 87% are anti-HBc(+) (Table 1). In the same areas, controls have much lower frequencies of HBsAg and anti-HBc. Even in the United States, patients with PHC have significantly higher frequencies of HBsAg and especially anti-HBc in their blood than do controls; i.e., serological evidence of persistent infection with HBV is significantly more common in the cases of PHC than in the controls (Szmuness 1978; R. L. Yarrish et al., in prep.).

3. As noted earlier, 80% or more of the cases of PHC arise in a liver already affected by cirrhosis and/or chronic active hepatitis. If chronic hepatitis and cirrhosis are steps on the way to the development of liver cancer, then case-control studies of these two diseases should also show higher prevalences of chronic infection with HBV in the cases as compared with appropriate controls. Studies in Africa, Taiwan, and Korea have demonstrated that almost all such patients are chronically infected with HBV (HBsAg[+] and/or anti-HBc[+]) (e.g., see Hann et al. 1979).

4. Since PHC is a cancer of the liver, one would expect to find signs of HBV infection in the hepatic tissues of patients with the disease. HBV proteins can nearly always be demonstrated in such tissues by histochemical stains or immunological techniques (Nayak et al. 1977). HBsAg and HBeAg are either not detected or found in only small quantities in the tumor cells themselves, but rather they are found in the nonmalignant cells adjacent to the expanding tumor. These proteins are not found in the livers of uninfected individuals or persons with anti-HBs in their serum.
### Table 1
Frequency of HBsAg and Anti-HBc in Patients with PHC and Controls

(a) Hepatitis-B surface antigen

<table>
<thead>
<tr>
<th>Country</th>
<th>PHC no. tested</th>
<th>PHC positive (%)</th>
<th>Controls no. tested</th>
<th>Controls positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greece</td>
<td>189</td>
<td>55.0</td>
<td>106</td>
<td>4.7</td>
</tr>
<tr>
<td>Spain</td>
<td>31</td>
<td>19.3</td>
<td>101</td>
<td>2.0</td>
</tr>
<tr>
<td>United States</td>
<td>34</td>
<td>14.7</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>Senegal</td>
<td>291</td>
<td>51.9</td>
<td>100</td>
<td>12.0</td>
</tr>
<tr>
<td>Mozambique</td>
<td>29</td>
<td>62.1</td>
<td>35</td>
<td>14.3</td>
</tr>
<tr>
<td>Uganda</td>
<td>47</td>
<td>47.0</td>
<td>50</td>
<td>6.0</td>
</tr>
<tr>
<td>Zambia</td>
<td>19</td>
<td>63.1</td>
<td>40</td>
<td>7.5</td>
</tr>
<tr>
<td>S. Africa</td>
<td>138</td>
<td>59.5</td>
<td>200</td>
<td>9.0</td>
</tr>
<tr>
<td>Taiwan</td>
<td>84</td>
<td>54.8</td>
<td>278</td>
<td>12.2</td>
</tr>
<tr>
<td>Singapore</td>
<td>156</td>
<td>35.3</td>
<td>1516</td>
<td>4.1</td>
</tr>
<tr>
<td>Japan</td>
<td>260</td>
<td>37.3</td>
<td>4387</td>
<td>2.6</td>
</tr>
<tr>
<td>Vietnam</td>
<td>61</td>
<td>80.3</td>
<td>94</td>
<td>24.5</td>
</tr>
</tbody>
</table>

(b) Antibody to hepatitis-B core antigen

<table>
<thead>
<tr>
<th>Country</th>
<th>PHC no. tested</th>
<th>PHC positive (%)</th>
<th>Controls no. tested</th>
<th>Controls positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greece</td>
<td>80</td>
<td>70.0</td>
<td>160</td>
<td>31.9</td>
</tr>
<tr>
<td>Spain</td>
<td>31</td>
<td>87.0</td>
<td>101</td>
<td>14.8</td>
</tr>
<tr>
<td>United States</td>
<td>33</td>
<td>48.5</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>Senegal</td>
<td>291</td>
<td>87.3</td>
<td>100</td>
<td>26.0</td>
</tr>
<tr>
<td>S. Africa</td>
<td>76</td>
<td>86.0</td>
<td>103</td>
<td>31.7</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>37</td>
<td>70.3</td>
<td>58</td>
<td>36.2</td>
</tr>
</tbody>
</table>

Only studies using radioimmunoassay or a test of equivalent sensitivity for HBsAg and in which controls were included are used. These data have not been corrected for age. Adapted from Szmuness (1978) and R. L. Yarrish et al. (in prep.).

5. If persistent HBV infection "causes" PHC, such infection should precede the occurrence of PHC. To test this hypothesis, it is necessary to identify the "healthy" chronic carriers of HBV and the controls who are not carriers and to follow up on the individuals in such groups over a period of several years to watch for the development of PHC. Two studies of this prospective design are currently in progress, one in Japan directed by K. Sakuma and his colleagues (pers. comm.) and another in Taiwan conducted by Beasley and Lin (1978). In the study in Japan, all employees of the Japan National Railway in Tokyo between the ages of 40 and 60 had a blood sample drawn as part of their annual physical examination. The workers found to have HBsAg in their blood were tested again 6 months later. If they were still positive, they were designated chronic carriers. A total of 341 such individuals were identified as compared with 17,843 HBsAg(-) noncarriers. Over the succeeding 6 months to 3.5 years, three cases of PHC occurred among the carriers, whereas none was observed among the noncarriers.

Beasley's study in Taiwan (Table 2) was conducted on male civil servants, also between the ages of 40 and 60. In a study similar to that conducted in Japan, 3000 carriers were identified. The controls were 3000 HBsAg(-) individuals matched by age, sex, and place of origin in mainland China or Taiwan. The subjects were followed-up for about 4 years. Eleven cases
Table 2
Prospective Study of the Development of PHC in Asymptomatic Male Government Workers in Taiwan

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>PHC</th>
<th>Total deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg(+) - chronic carriers</td>
<td>3000</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Matched controls</td>
<td>3000</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

Eleven cases of PHC developed in the group, all of which were in the carrier group. The carriers and controls were followed up prospectively for 4 years (Beasley and Lin 1978).

occed during the follow up, and all of the cases developed in the chronic carriers. These two studies provide very strong support for the hypothesis that chronic infection with HBV is etiologically related to PHC.

6. As stated previously (point 3), PHC usually develops in a liver affected by cirrhosis and/or chronic hepatitis. Some investigators have argued that hepatotoxic agents (e.g., alcohol and liver parasites such as Schistosoma mansoni and Clonorchis sinensis) that cause cirrhosis are associated with an increased risk of developing PHC and that HBV is “just” one more hepatotoxic agent. Therefore, a particularly rigorous test of the hypothesis that chronic infection with HBV imparts an increased risk of PHC beyond its role in the production of cirrhosis is to compare the incidences of PHC in patients with cirrhosis of the liver who are or are not chronic carriers of HBV. Such a study is being carried out in Japan by Obada (1977). Beginning in April 1973, patients with cirrhosis were categorized as HBsAg(+) or HBsAg(-), carefully evaluated to be sure that they did not have PHC on admission to the study, and then followed-up for the development of PHC. As shown in Table 3, the patients who were HBsAg(+) were at about four times the risk (during a 3- to 4-year follow-up period) of developing PHC as were the HBsAg(-) patients.

7. In populations where HBV is endemic, there is good evidence that many of the chronic carriers arise as a result of infection transmitted from their mothers early in life (at the time of delivery, in the period after birth when the mother and child have considerable close contact, or, perhaps, prenatally), i.e., the mothers themselves are chronic carriers and offspring born at times when the mothers are infectious are likely to become chronic carriers.

Table 3
Development of PHC in Japanese Patients with Cirrhosis of the Liver

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>Developed PHC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg(+)</td>
<td>30</td>
<td>7 (23.3)</td>
</tr>
<tr>
<td>HBsAg(-)</td>
<td>85</td>
<td>5 (5.9)</td>
</tr>
<tr>
<td>Total</td>
<td>115</td>
<td>12 (10.4)</td>
</tr>
</tbody>
</table>

These patients were followed up for 1 to 4.5 years. Nearly 25% of the patients in whom the cirrhosis was associated with HBV, but only 6% of those presumably due to other causes, developed PHC (Obada et al. 1977).

*Number in parentheses indicates the percentage of patients who developed PHC.
HBV and Liver Cancer

(Blumberg 1972). The potential infectiousness of the mothers appears to be associated with the presence of intact hepatitis-B virions, DNA polymerase, and HBeAg in their peripheral blood. Among members of a population, individuals infected at birth will have been chronic carriers of HBV longer than chronic carriers of similar age infected later in life. Therefore, if the duration of being a chronic carrier is related to the likelihood of developing PHC, one could predict that the mothers of patients with PHC would be more likely to be chronic carriers than the mothers of control individuals of similar age who do not have PHC. Larouze et al. (1976) conducted such a study in Dakar, the capital of Senegal in West Africa. We found that about 70% of the mothers of patients with PHC were HBsAg(+) as compared with only 6% of the mothers of controls. Even when mothers of PHC patients were compared with mothers of HBsAg(+) carriers without cancer, the mothers of the cancer patients were significantly more likely to be HBsAg(+). In collaboration with Chong Yong Kim of the National University Hospital and our colleague Hie-Won Hann, we have carried out similar studies in Seoul, South Korea, and our findings are consistent with the African studies (Hann et al. 1979) (Table 4).

If chronic-carrier mothers give birth to children who become chronic carriers and such children have an increased risk of developing PHC, then other children born to the same mothers should also have an increased risk of becoming carriers and developing chronic liver diseases including PHC. Studies by Drs. Hann, Kim, and our group in South Korea support this notion. About 60% of brothers and 40% of sisters of patients with PHC are HBsAg(+); the brothers appear to have an increased prevalence of chronic active hepatitis, cirrhosis, and PHC.

We have also detected a paternal effect. Spouses of chronic carriers generally have a very high prevalence of anti-HBs (70-90% in our studies). In Korea and Senegal, fathers of patients with PHC or other chronic liver diseases had significantly lower frequencies of anti-HBs than men of similar age in the general population, despite chronic exposure to carrier wives and/or carrier children. The nature of this paternal factor is not clear at this time.

8. Most studies of the relationship of viruses to cancer have been done in experimental animals or tissue-culture systems. It is thought that the genomes of such viruses become integrated into the genome of the host cell and that the product of a viral gene is required to produce malignant transformation of the cell.

Summers et al. (1978b) has tested this hypothesis. He isolated HBV DNA and, with the use of DNA polymerase from Escherichia coli, made radioactively labeled copies of the HBV DNA that in turn could be used as probes to see whether viral DNA was present in PHC tissue, i.e., would hybridize with DNA in the liver tumor cells. His studies demonstrated viral DNA in the liver cells of patients with PHC who showed evidence of HBV infection in their blood. The viral DNA, however, was located within protein cores and did not appear to be integrated into the genomes of the tumor cells. Because it is not certain that a given block of tissue removed at autopsy is free of nontumor cells and blood, it is difficult to know whether the viral DNA was extracted from malignant or nonmalignant hepatocytes. In any event, Summers was unable to demonstrate integration of the HBV genome into malignant or nonmalignant hepatocyte genomes.
### Table 4
Frequency of HBsAg, Anti-HBe, and Anti-HBs in Mothers of PHC Patients Compared with Controls

<table>
<thead>
<tr>
<th></th>
<th>Senegal</th>
<th></th>
<th></th>
<th></th>
<th>Korea</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHC</td>
<td>controls</td>
<td>PHC</td>
<td>controls</td>
<td>PHC</td>
<td>controls</td>
<td>PHC</td>
<td>controls</td>
</tr>
<tr>
<td></td>
<td>no. tested positive (%)</td>
<td>no. tested positive (%)</td>
<td>no. tested positive (%)</td>
<td>no. tested positive (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg</td>
<td>28</td>
<td>72</td>
<td>28</td>
<td>14</td>
<td>9</td>
<td>44</td>
<td>114</td>
<td>4</td>
</tr>
<tr>
<td>Anti-HBe</td>
<td>28</td>
<td>72</td>
<td>28</td>
<td>14</td>
<td>9</td>
<td>100</td>
<td>46</td>
<td>52</td>
</tr>
<tr>
<td>Anti-HBs</td>
<td>28</td>
<td>11</td>
<td>28</td>
<td>54</td>
<td>9</td>
<td>44</td>
<td>114</td>
<td>45</td>
</tr>
</tbody>
</table>

For Senegal, the controls were mothers of individuals who did not have PHC, but who were usually carriers of HBsAg. For Korea, the controls were other women in the same population (Larouzé et al., 1976; Hann et al., 1979).
Recently, Marion and Robinson (this volume) studied a unique tissue-culture preparation originally developed by Alexander of South Africa from the liver of an African patient who had died of PHC. He found that part of the genome of HBV was integrated in three places into the DNA of the chromosomes of the liver cells growing in the tissue culture. It is not clear at present how this relates to the studies on liver cells from biopsies and autopsies.

9. The existence of primary carcinoma of the liver associated with a virus similar to HBV in *Marmota monax* is an additional piece of evidence supporting the hypothesis. This is discussed in detail by Snyder and Summers (this volume) but will be described briefly here.

Robert Snyder has trapped in the wild and maintained at the Philadelphia Zoological Garden for the past 18 years a colony of Pennsylvania woodchucks (*M. monax*). During this period, he has performed postmortem examinations on 102 woodchucks; 23 of these had primary liver cancer, and three had chronic active hepatitis. He further noted that, as in humans with PHC, the tumors in the animals were usually associated with chronic hepatitis and, sometimes, cirrhosis.

Several years ago, we examined the sera of some of these animals for the presence of HBsAg but did not detect it. Summers studied these sera again, this time with more rewarding results (Summers et al. 1978a). He based his investigation on the hypothesis that viruses of the same class as HBV (which we had called icrons; see below) would have similar nucleic acid structures and similar DNA polymerases; i.e., they would contain a circular double-stranded DNA genome with a single-strand region and a DNA polymerase capable of filling in the single-strand region to make a fully double-stranded circular DNA. He assayed serum samples from the woodchucks for particles containing DNA polymerase with this activity; about 15% of the serum samples had such particles. Summers and his colleagues went on to study three animals in great detail; two that had died of liver cancer and one of a myocardial infarction. They found that the two animals with liver tumors had particles in their sera very much like the three types of particles associated with HBV; the animal without tumor did not have such particles. The viral DNA was, as predicted, very similar to HBV in size and structure.

Recently, Werner et al. (1979) found cross-reactivity between the surface (WHsAg) and core (WHcAg) antigens of the woodchuck hepatitis virus (WHV) and the comparable antigens on human HBV. Antiserum against HBCAg precipitates the cores of WHV and antisera against WHV cores (anti-WHC) precipitates HBCAg. There is also cross-reactivity of the surface antigens, but it is less than that for the core antigens. Anti-HBs will not precipitate WHsAg or vice versa, but antibody to WHsAg (anti-WHS) will agglutinate red blood cells coated with HBsAg, and anti-HBs antibodies will agglutinate erythrocytes coated with WHsAg.

A carrier state for WHV in *M. monax* that appears to be analogous to the HBsAg carriers in humans has been found. The frequency of carriers among Pennsylvania and New Jersey woodchuck populations is 10–20%, similar to that found for HBV in several human populations.

Liver cancer in the woodchuck is not what is generally thought of as a laboratory model of a human disease, i.e., it was not designed or "created" by an investigator for his own purpose, rather it is a naturally occurring disease.
related to a naturally occurring virus, both of which have a remarkable number of features in common with their human counterparts. It may be possible by careful study of the relationship of WHV to primary liver cancer in the woodchuck to understand the mechanisms that relate HBV to human liver cancer. For example, it appears that the woodchucks that develop PHC are chronic carriers of WHV, and it is likely (although not proved) that animals that develop antibody to the surface antigen of WHV are protected from PHC. Thus, the factors that result in either of these two polar responses to WHV can be investigated and identified. Factors that appear relevant in the development of the chronic carrier state and PHC in the woodchuck can be studied epidemiologically in humans, and, vice versa, factors associated with PHC from epidemiological studies in humans can be investigated experimentally in woodchucks.

Sequential Events in the Development of Liver Cancer

The preceding nine pieces of evidence, in addition to supporting the hypothesis that chronic infection with HBV is required for the development of PHC, also provide insight into a sequence of events that may begin at birth or before birth and progress ultimately to cancer (Fig. 1). HBV is transmitted from an infected mother to her child early in life. The child becomes a chronic carrier of the virus and over a period of many years develops a chronic inflammatory disease of the liver, chronic active hepatitis; this leads to regeneration of liver tissue and proliferation of fibrous tissue (cirrhosis); and finally, by mechanisms that are currently unclear, cancer can arise from the regenerating liver tissues. Possible models for the development of cancer in these situations are described below.

This sequence of events can be considered the life history of one person who develops PHC. On a population basis, however, the sequence is more like a pyramid. A large number of chronic carriers (5–20% of the population) are present in endemic areas as a result of maternal and other means of transmission of the virus. Some of the chronic carriers develop chronic active hepatitis, but the majority do not; some proportion of the individuals with chronic active hepatitis develop cirrhosis, but most do not; and finally, some of the individuals with cirrhosis develop PHC, but again most (~75% from the Japanese study) do not. Therefore, it is important to investigate which factors lead to progression from one stage to the next and which lead to arrest of the process or even reversal.

Figure 1
Scheme of pathogenesis of primary hepatocellular carcinoma.
Inherited Susceptibility, Polymorphisms, and Hepatitis-B Subtypes

Early family studies on the distribution of the Australia antigen (the term initially used for what is now known as hepatitis-B surface antigen) were consistent with the hypothesis that the ability to become persistently infected, i.e., to become a carrier, segregated as if it were an autosomal recessive trait (Blumberg et al. 1969). Subsequent studies, in general, have supported the notion of some genetic control, but they also show that the simple genetic model is not tenable. There is a striking male/female difference in the distribution of the carrier state (more males than females). There is a change in frequency of the carrier state with age; and maternal transmission, as already noted, may be common. Furthermore, it is clear that there is direct horizontal transmission, i.e., from one person to a genetically unrelated second person. The genetic hypothesis could be modified from expectation in view of these differences (i.e., partial penetrance, expressivity, sex effects, etc.). It would be better, however, to develop a new genetic-environmental hypothesis on the basis of HBV epidemiological and virological information, but this has not yet been done.

The early population and family studies of hepatitis-B carriers also were consistent with its description as a serum polymorphism (London et al. 1972). The identification of Australia antigen (HBsAg) as a surface component of a virus does not diminish the value of the polymorphism concept. It is useful to view the responses to infection with HBV (i.e., development of the chronic carrier state or transient infection with production of anti-HBs) not only in a conventional manner, but also as a transfusion or transplantation reaction. Studies on renal transplantation are an example of this.

HBV appears to have only a small amount of nucleic acid, probably only sufficient to code for a few proteins. Some of the coat (and possibly other portions of the virus) may be contributed by the host. (Recent successes in cloning of HBV DNA in bacteria will help to identify the proteins that are coded by viral genes.) Millman et al. (1971) reported that the surface antigen contains material with antigenic specifications in common with serum proteins, including IgG, transferrin, albumin, and β-lipoprotein. These observations were confirmed by Neurath et al. (1974). Recent evidence indicates that HBsAg has specific binding sites for albumin and that HBeAg binds tightly to IgG. Receptors for other host proteins may also be present. If this were true, then the antigenic makeup of the virus would be, at least in part, a consequence of the antigenic characteristics of the host from which it came. The responses to HBV of the putative host may be dictated in part by the nature of the “match” between the antigens of the host and virus, i.e., the virus acts as if it were a polymorphic human antigen. If person A is infected with HBV particles containing proteins antigenically very similar to his own, then he will have little immunologic response and will tend to develop a persistent infection with the virus. On the other hand, if the proteins of the agent are antigenically different from his, he will develop an immune response to the virus (i.e., anti-HBs) and will have a transient infection. During the course of infection in person A, new particles will be synthesized that contain the antigenic characteristics of A. In turn, person A can infect person B and the same alternatives will present themselves. If the relevant proteins of person B are antigenically similar to the antigens of person A and the antigens on HBV supplied by host A, then person B could develop a persistent infection. If they are different, then antibodies can form, as described
above. A likely possibility is that the virus has complex antigens: Some may match the putative host and some may not, and both persistent infection and development of antibodies may occur. The persistent antigens and the antibodies in the same individual would have different specificities; this occurrence was described earlier.

**Subtypes of HBsAg**

The distribution of the subtypes or strains of HBV is consistent with the distribution of polymorphic traits. Levene and Blumberg (1969) found that different subtypes of Australia antigen could be identified by rabbit immunizations with appropriate surface antigens. LeBouvier (1971) and others enlarged and systematized the findings. All surface antigens (HBsAg) have a common antigen ("a"). There are two sets of antigens, d and y and w and r, that appear to have an "allelic" relationship to each other as though they were controlled by sets of genes at two different loci. A particular viral surface antigen may be either d or y, but rarely both and rarely neither, and w or r, but rarely both and rarely neither. Other specificities have also been reported, but these have not yet been described in great detail.

There are specific geographic localizations of the HBsAg subtypes. For example, in North America and northern Europe, the d variant is very common but the y less so. The r subtype is found in infected people in Asia and Oceania, in people elsewhere who originate from the areas of high frequency, but rarely in people from the Americas, Europe, and elsewhere. In Japan, the ancient peopling of the island is reflected in the current distribution of subtypes. The populations of the Ryukyu Islands (south of Japan's main islands) and southern Japan have subtypes similar to those of Indonesians and Malaysians, which reflect migrations from these regions. The populations of the northern parts of the Japanese islands reflect the long-ago migrations from Central Asia (through Korea) and regions of the Asian mainland to the north of Japan.

**Sex Differences in Response to HBV**

There are a large number of human male-female differences with respect to their interactions with HBV.

The differences in the relative numbers of males and females vary in the postulated sequence of conditions leading from early persistent infection with HBV to cancer of the liver (Fig. 1). If a mother is a carrier of HBV, then there is about an equal chance that her sons and daughters will become infected at birth. However, it is somewhat more likely (ratio ~1.5:1) that the sons will remain infected and become carriers and develop chronic active hepatitis. More males than females (ratio ~3:1) will develop cirrhosis of the liver, and an even greater proportion (ratio ~6:1) will develop primary hepatic carcinoma (Sherlock 1975). Thus, in this unfortunate progression toward cancer, only a portion of individuals entering each stage will go on to the next, and the ratio of males approximately doubles with each progressive stage.

Once infected with HBV, males are more likely to remain persistently infected and become carriers; females are more likely to be transiently infected and then develop antibody to the surface antigen. Our most quantitative data come from a 5-year study of patients with end-stage kidney disease treated in an artificial
kidney unit (London and Drew 1977). In this unit, patients had a high probability of infection with HBV. Males had a 68% chance of remaining HBsAg(+) after an initial infection with HBV as compared with a 33% risk for females. Conversely, females had a 55% chance of losing the antigen and developing anti-HBs after infection as compared with a 25% chance for males. Szmuness et al. (1978) have confirmed these observations with patients from a large number of dialysis units.

There have been additional observations on sex differences and responses to HBV. We studied patients in Philadelphia with end-stage kidney disease who had received renal transplants (London et al. 1977). If the recipients had anti-HBs, their kidney grafts had a significantly shorter survival than recipients who were HBsAg carriers or recipients who had not been infected. The shortest graft survival was in a subgroup of patients with anti-HBs that received grafts from male donors. Grafts from female donors into anti-HBs(+) recipients did not have a shortened survival. (Most “donors” of cadaver kidneys are males. This is apparently due to the practice of obtaining kidneys from those who have died as a result of trauma and the higher incidence of deaths due to trauma in males as compared with females.) Since the publication of our report, seven other groups have examined their transplant populations. Five groups have observed shorter graft survival in patients with anti-HBs and two have not. The differences may possibly be explained by the manner of exposure to HBV. Our patients, and those of the groups whose results are consistent with ours, appear to have been infected by repeated exposure to chronic carriers in hemodialysis clinics. The reports by groups with results that differ from ours suggest that their patients with anti-HBs were infected in the outside community and not in a dialysis clinic. This implies that HBV strains (HBsAg subtypes) endemic in dialysis clinics may be antigenically different from those in the general community, and the responses to such subtypes may have different consequences. We have shown that the HBsAg subtypes present in carriers in the dialysis unit, where most of our patients were infected, differ from those present in carriers outside the unit but in the same geographic location. This suggests that the immune response to a certain HBsAg subtype or antigenic determinant present on some but not all HBV strains may be the factor associated with kidney-graft rejection.

The third sex difference associated with response to HBV is related to another kind of “graft.” We studied the sex ratio at birth of offspring according to the response of parents to HBV infection (Drew et al. 1978). In a village in Greek Macedonia, we found that there was a high proportion of boys born to couples in which either the mother or father was a carrier. Among couples in which the mother had anti-HBs and the father was not a carrier, there was a significantly lower proportion of male children. We have found the same trend in populations from Papua New Guinea and Greenland. Extensive studies of this kind have not been carried out as yet by other investigators.

These observations on sex differences could be explained by a variety of biological mechanisms. One hypothesis (which can be tested) is that there is cross-reactivity between HBsAg and a male-associated antigen, i.e., the antigen may be present in males but never, or only rarely, in females. If HBsAg cross-reacts with a male-associated antigen, males would be more likely to recognize HBsAg as “self” and therefore would remain HBsAg(+) persistently. Females, however, would be more likely to recognize HBsAg as “foreign” and produce anti-HBs. This would result in the observed predominance of males among chronic carriers of HBV in dialysis patients and other populations. In kidney-
transplant patients, tolerance to HBsAg in kidney-graft recipients would result in longer survival of male tissues, whereas recipients with anti-HBs would react to male antigens on renal allografts, resulting in early rejection of grafts from male donors.

Similarly, we can speculate that tolerance to HBsAg (reflected in the maintenance of HBsAg[+]anti-HBs[−] status) in pregnant women would result in a lack of sensitization against male tissues developing within them and therefore good survival of male fetuses. Anti-HBs in women, however, could react with male antigens and perhaps hinder fertilization by sperm bearing a Y chromosome or increase the probability of spontaneous abortion of male fetuses. HBsAg(+) males would have HBsAg in their semen that, conceivably, could protect Y-bearing sperm from the effects of anti-HBs or other "anti-male" antibodies in their spouses' reproductive tracts.

We can now propose a hypothesis that brings together the data on sex differences in response to infection with HBV with the development of chronic liver disease and cancer. The contrasting courses of HBV infections can be characterized as a female-type reaction or a male-type reaction. In the female-type reaction, the sequence of events appears to be typical of many noncytopathic viral infections: (1) virus binds to receptors on hepatocytes; (2) DNA of the HBV enters the hepatocyte; (3) virus replicates in the hepatocyte; and (4) virus is produced. Replication of HBV does not appear to cause cell death; most cell damage probably results from an immune response to antigenic determinants on virus-infected cells. Doherty et al. (1976) have demonstrated (with other viruses) that destruction of virus-infected cells is carried out by cytotoxic T lymphocytes with dual specificities. Such T cells recognize viral antigens and self-antigens (HLA in humans and H-2 in mice) on the surface of the infected cell of the host. There is also an immune response to virus released from infected cells. Thus, infection of females with HBV is associated with acute, but self-limited, liver damage; self-limited because the anti-HBs antibodies produced by immune-responsive B lymphocytes clear the blood of circulating virus and T lymphocytes (probably) destroy virus-infected cells in the liver. The liver is able to regenerate, and after a few months it returns to a functionally and structurally normal state.

In the male-type reaction, the first part of the infectious process is probably similar to that in females, i.e., hepatocytes are infected and virus is produced. Because of the postulated cross-reactivity between a determinant on HBsAg and a male-associated antigen, however, the immune-responsive cells do not recognize either the virus or the virus-infected cells as "foreign." Therefore, virus continues to be produced with only minor damage to liver cells. However, this is not the only kind of reaction possible in males. As already described, the viral antigens are polymorphic (HBsAg subtypes d, y, w, r, etc.) (see, for example, LeBouvier 1971) and the postulated male-associated cell-surface antigen may also be polymorphic, i.e., some males may cross-react with the HBsAg male-type antigen and some may not. A given human male could be infected with HBV with an antigenic determinant (or determinants) similar or dissimilar to antigens on his own cells. If the antigens are dissimilar, his immune system would recognize them as foreign and the female-type reaction would occur.

A further consequence of the polymorphic nature of both the virus and the human host is that situations of partial recognition and partial dissimilarity could occur. Some antigens on the virus would not be shared by the host and would be recognized as foreign, but another might cross-react. This could result
in a modified immune response, i.e., not sufficient to clear HBV from the
circulation or HBV-infected hepatocytes from the liver. An immune response in
the presence of persistent HBV infection could result in continuing damage to
infected hepatocytes (chronic hepatitis and cirrhosis).

How does this biological model account for the occurrence of chronic liver
disease in females? In addition to the polymorphism of the sex-related antigen, it
is postulated that there are antigenic equivalents in the host to the non-sex-
related antigens of the HBsAg (the “autosomal” antigens) that are also
polyorphic. The same types of match, mismatch, and combination of match and
mismatch may occur with these antigens with the consequences already described
for the sex-related antigen. Since the predominance of disease in males is so
striking, the model allows for a pathogenetically greater effect of the sex-related
antigens than the autosomal antigens. Also, the male-associated antigen may
occasionally occur in females.

An animal model for alternative pathologic outcomes of a nonlytic viral
infection is lymphocytic choriomeningitis (LCM) in mice. Infection with LCM
virus in adult mice follows the scheme described for HBV infection in females.
The mice develop an acute encephalitis mediated by cytotoxic T cells and usually
die. In contrast, LCM infection of neonatal mice leads to persistent infection
with virus and a continuing immune response. In such mice, most of the cell
damage is done by LCM antigen-antibody immunocomplexes. Immuno-
complexes are present in HBV-associated chronic active hepatitis in humans,
but whether liver-cell damage is caused by immunocomplexes, cytotoxic T cells,
or both is unclear.

Biological Models of Cancer of the Liver

The accepted model for how viruses cause cancer is that nucleic acid from the
virus is integrated into the genome of the host cell. An integrated viral gene then
produces malignant transformation of the cell, and all of the progeny of that cell
are cancer cells. Although this is an attractive hypothesis, it does not deal with
the epidemiological and clinical findings, e.g., sex differences in the incidence of
PHC, the background of chronic liver disease, and the long incubation period.

We suggest another model that attempts to account for the clinical and
epidemiological observations. As pointed out previously, the liver is a
regenerative organ. The stimulus for hepatic cells to divide is death or removal of
hepatocytes. In the presence of continuous liver-cell destruction, there would be
continuous liver-cell regeneration. One would expect that, as a result of random
mutation or exposure to environmental carcinogens, the dividing hepatic stem
cells would give rise to occasional mutant hepatocytes, some of which would be
malignant. The malignant clones that would have the best chance of survival
(becoming clinically apparent cancers) would be those that lacked receptors for
HBV or, once infected, lacked the ability to express HBV antigens and thus
would escape damage from immune responses to antigens on virus-infected
cells. As noted above, in histopathological studies HBsAg is usually found in
lesser quantities in the tumor cells and in larger amounts in the cells immediately
surrounding tumor foci. Although development of liver cancer in individuals
with persistent HBV infection and chronic liver disease can be considered a
failure of the immune system to prevent the tumor, this differs from the
imunosurveillance hypothesis of cancer, since it is not a failure of the immune
system to recognize tumor antigens, but an enhanced chance of survival of
malignant cells over their nonmalignant neighbors because of a relative lack of viral antigens on the tumor cells. The model can be viewed as an “internal polymorphism” of liver cells with respect to response to infection with HBV, analogous to the polymorphism of response to HBV infection in human populations.

This model for the pathogenesis of primary liver cancer accounts for the association of persistent HBV infection with both chronic liver damage and PHC. It also accounts for the decreased quantity of HBV antigens in hepatic cancer cells and their presence in nonmalignant cells of tumor-bearing livers. Finally, it offers an explanation of sex differences in the incidence, prevalence, and mortality of HBV-associated chronic liver diseases and PHC.

Balance between Malignant and Normal Cells

There is an interesting conceptual consequence of the reasoning used to develop this biological model of the pathogenesis of primary hepatocellular carcinoma. Most models of carcinogenesis imply that the causative agent affects a previously normal cell by influencing its genetic mechanism to change it into a cancer cell. The assumption is that any cell that has been transformed into a cancer cell will give rise to a malignant tumor. The present model implies that the effect of the hepatitis virus is to slowly eliminate normal cells, with a concomitant increase in cells that have the potential to develop into cancers. The immediate transforming event, which could be a spontaneous mutation, a carcinogen (e.g., aflatoxin)-induced mutation, or even integration of an HBV genome (which, according to recent work by Robinson et al. [this volume], may occur under certain conditions), is insufficient to produce a clinical cancer. Therefore, under normal conditions, there is a balance between (1) cells that are unlikely to become cancerous and (2) cells that are either already cancerous, but kept in “control” by normal neighbor cells, or more likely to give rise to cancer cells during subsequent divisions. According to this model, the HBV infection upsets this balance because of its differential effect on the malignant and nonmalignant cells, leading to greater destruction of nonmalignant cells.

Prevention of Chronic Liver Disease and PHC

We believe that the data that have been presented and other studies now being done support the hypothesis that persistent infection with HBV is necessary for the development of chronic liver disease and PHC. If present and future studies continue to support the hypothesis, then it would follow that prevention of infection with HBV and/or modification of the host response after infection could lead to a decrease in the incidences of these deadly diseases. To effect control, it is necessary to know the modes of transmission and factors related to host response. HBV is a very adaptive organism, and several modes of transmission are known. This could be construed as unfortunate since it might be difficult to control all the methods of transmission. Another view is that the existence of many infection routes allows for multiple strategies for control, i.e., there are numerous vulnerable places in the scheme of pathogenesis where preventive techniques could be applied.

HBV can be transmitted directly by blood transferred from an infected individual to an uninfected one, i.e., transfusion, reuse of contaminated needles, tattooing, etc. It is transmitted readily from husband to wife and probably by
venereal means. It is found in the saliva and presumably could be spread by the respiratory route. Maternal transmission has already been discussed. It is usually assumed that the fecal-oral route (i.e., by fecal contamination of water and food) is not a major method of transmission because the virus cannot be regularly detected in the feces of infected persons. However, there are various agents in feces, including certain bacteria, that alter the antigenicity of HBsAg to make it undetectable. Hence, poor sanitation could also be responsible for transmission. At least two potential insect vectors (mosquitoes and bedbugs) have high field-infection rates for HBsAg and they may be an important means of transmission.

The vaccine against HBV that we introduced (Millman and Blumberg 1978), if proved safe and effective, could have an important role in prevention strategies. Other public health measures useful in controlling infectious diseases can also be utilized. These measures can be justified not only for the prevention of PHC, but also for the prevention of chronic liver disease, a major cause of morbidity and mortality in areas where HBV is common.

It should be possible to determine in a relatively short time whether prevention programs are effective by measuring changes in the risk factors brought about by the program. This could have the effect of encouraging similar investigations for other common cancers.

The Icron

Early in our studies it was recognized that HBV had unusual characteristics of structure and behavior. As a heuristic device, we suggested the group term Icron to designate HBV and other infectious agents that might be found in the future with characteristics sufficiently similar to be grouped with HBV. It is a proper name; however, the name itself does not carry any functional significance. Rather, as the characteristics of Icrons become known, they will impart a meaning to the name.

Table 5 lists the characteristics of HBV, most of which have been described in the preceding pages. These characteristics can be compared with those of known or newly discovered viruses. A major value of such a listing is that it suggests hypotheses generated from knowledge of one virus (HBV) that can be tested in other viruses. WHV is the second member of the Icron group and shows many, but not all, of these characteristics. This approach has some similarities to the work of botanical systematists who study numerous characteristics of apparently similar plants and compare them with a type specimen, usually the first found of a species.

The objective of this exercise is to identify other viral agents in addition to WHV that have properties in common with HBV; in particular, those associated with commonly occurring cancers. If these are found, then it may be possible to develop preventive methods analogous to those proposed for HBV and cancer of the liver.

ACKNOWLEDGMENTS

This work was supported by U. S. Public Health Service grants CA-06551, RR-05539, and CA-06927 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania.
**Table 5**
Comparison of HBV and WHV Icers

<table>
<thead>
<tr>
<th>Particles</th>
<th>HBV</th>
<th>WHV</th>
</tr>
</thead>
<tbody>
<tr>
<td>virion</td>
<td>~40-50 nm dia.</td>
<td>~40-50 nm dia.</td>
</tr>
<tr>
<td>small surface antigen</td>
<td>~20-25 nm dia.</td>
<td>~25 nm dia.</td>
</tr>
<tr>
<td>elongated surface antigen</td>
<td>~20-25 nm dia., various lengths</td>
<td>~25 nm dia., various lengths</td>
</tr>
<tr>
<td>(core)</td>
<td>~27 nm dia.</td>
<td>~27 nm dia.</td>
</tr>
<tr>
<td>DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>circular</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>double- and single-stranded</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA polymerase present</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>no. of nucleotides</td>
<td>~3182</td>
<td>~3400</td>
</tr>
<tr>
<td>homology with HBV</td>
<td>100%</td>
<td>3-5%</td>
</tr>
<tr>
<td>Antigens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>surface</td>
<td>+ HBsAg</td>
<td>+ WHsAg 0.1-1% cross-reacting with HBsAg</td>
</tr>
<tr>
<td>core</td>
<td>+ HBsAg</td>
<td>+ WHcAg 5-10% cross-reacting with HBsAg</td>
</tr>
<tr>
<td>“e” antigens</td>
<td>+ present in core</td>
<td>+ present in core</td>
</tr>
<tr>
<td>subtypes</td>
<td>a common; d, y, w,</td>
<td>a common; d, y, w,</td>
</tr>
<tr>
<td></td>
<td>r, others</td>
<td>r, others</td>
</tr>
<tr>
<td>Responses to infection</td>
<td>+ 0.1-~20% in human</td>
<td>+ 10-20% in Pennsylvania, population Maryland, and New Jersey; M. monax</td>
</tr>
<tr>
<td>carrier state</td>
<td>population</td>
<td></td>
</tr>
<tr>
<td>Clinical conditions</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>----------------------------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>acute hepatitis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>chronic hepatitis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cirrhosis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>primary hepatocellular</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>carcinoma</td>
<td>+</td>
<td>+</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Male-female ratios</th>
<th>M &gt; F</th>
<th>M &gt; F</th>
<th>M &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>carriers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acute hepatitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chronic hepatitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>primary hepatocellular</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transmission prevention</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>transfusion</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>oral respiratory</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>bedbugs</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>feces</td>
<td>not clear</td>
<td>not clear</td>
<td>not clear</td>
</tr>
<tr>
<td>maternal</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>&quot;cultural practices&quot;</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>vaccine</td>
<td>+</td>
<td>+</td>
<td>+</td>
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+ effectiveness now being tested
REFERENCES


ASSOCIATION OF HEPATITIS B VIRUS INFECTION WITH HEPATOCELLULAR CARCINOMA IN AMERICAN PATIENTS

Robert L. Yarrish, Barbara G. Werner and Baruch S. Blumberg
Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pa. 19111, USA.

Thirty-four patients from the Philadelphia area with hepatocellular carcinoma (HCC) were matched with colon cancer patients, lung cancer patients and blood donors according to age and sex. Sera from the four groups were tested to determine the prevalence of hepatitis B surface antigen (HBsAg), antibody to HBsAg (anti-HBs), and antibody to hepatitis B core antigen (anti-HBc). Five of the HCC patients (14.7%) and none of the controls were positive for HBsAg. At least one of the three serologic markers of hepatitis B virus (HBV) infection was found in 51.5% of the HCC patients, 5.3% of the colon cancer patients, 11.1% of the lung cancer patients, and 10.7% of the blood donors. Twelve of the seventeen seropositive HCC patients (70.6%) were positive for anti-HBc alone, while all of the seropositive lung cancer patients and donors were positive for anti-HBs alone. Sera positive for any HBV marker were also tested for e antigen (HBeAg) and its antibody (anti-HBe). Four of the HCC patients (23.5% of the seropositives) had anti-HBe, while none of the sera tested had HBeAg. A history of alcoholism did not appear to influence HBV seropositivity in the HCC patients. This study supports the hypothesis that HBV infection is closely associated with HCC even in areas where both conditions are uncommon. The wide disparity between seropositivity for HBsAg and anti-HBc in the HCC patients is an unusual feature, for which an age effect may be the best explanation.

Since the discovery of the Australia antigen, many investigators have used serologic, pathologic, and epidemiologic methods to study the association between hepatitis B virus (HBV) infection and hepatocellular carcinoma (HCC). Their findings, which have been reviewed comprehensively by Blumberg et al. (1975) and Szmuszas (1978), strongly suggest that a significant association between HBV infection and HCC does exist. Most of the data supporting this association, however, come from populations in Africa and Asia which have relatively high prevalences of these two conditions. Demonstrating a similar association in areas such as the United States, where HBV infection and HCC occur much less frequently, has been more difficult; and investigation in these areas has probably been discouraged to some extent by the findings of studies done when methods for detecting HBV infection were in their earliest stages of development (Alpert and Isselbacher, 1971; Smith and Blumberg, 1969; Prince et al., 1970).

More recently, two studies of HCC patients from California have used serologic tests and special stains for HBV antigens in liver tissue to show a frequency of HBV infection of over 70% in those patients showing non-alcoholic liver disease, while the rate of infection in alcoholic patients was much lower (Peters et al., 1977; Omata et al., 1979). A study of 24 British Caucasian HCC patients showed evidence of HBV infection in 67% but found no significant bias in favor of non-alcoholic patients (Bassendine et al., 1979). None of the above studies, however, used other cancer patients as controls. A recent study from Greece which did use such controls suggested that HBV infection was a significant risk factor for the development of HCC (Trichopoulos et al., 1978), but the applicability of these findings to American and other European populations is uncertain since Greece has a prevalence of HBV infection approaching that of many Asian and African countries (Hadjizyannis, 1974). In the present study we have examined sera from 34 HCC patients from the Philadelphia area. To control for the possibility that hospitalization and severe illness may themselves be risk factors for the development of HBV infection, we have age- and sex-matched our patients with two groups of patients having other malignancies as well as with a group of presumably healthy blood donors.

MATERIAL AND METHODS

Study and control populations

Sera used for the study were collected from several Philadelphia hospitals between 1968 and 1977 and stored at $-20^\circ$C in the blood collection of the Institute for Cancer Research. The use of sera and patient information was approved by the Institutional Review Committee in December, 1976. Diagnoses were obtained from physicians who supplied the specimens. The diagnosis of HCC was verified by histologic examination in all cases except one, in which the diagnosis was made by an elevated serum alpha-fetoprotein and typical clinical findings and course. Demographic information was obtained from hospital records or, when possible, from patient interview.

Sera from the HCC patients were matched according to age and sex with sera from three control groups: (1) patients with carcinoma of the colon; (2) patients with carcinoma of the lung; and (3) blood donors from a local general hospital. Control sera were selected so as to minimize age differences between HCC patients and controls, and a maximum age difference of 5 years was allowed in matching. Within the limits of the material available, each HCC serum was matched with sera from two blood donors. In several instances where two separately drawn specimens from an HCC patient were available, the patient was matched with two colon and

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Received: August 13, 1980.

Serologie

Proben wurden für HBsAg durch Radioimmunoassay (Austria II, Abbott Laboratories, North Chicago, Ill.), und alle Positiven wurden durch counter-immunoelektrophoresis (CIE) gegen Referenzserum enthaltend anti-HBs bestätigt.


Ergebnisse

HBsAg wurde signifikant häufiger in der HCC-Gruppe als in den Kontrollen gefunden. In Tabelle II, fünf der 34 HCC-Patienten (14.7%), aber keiner der Kontrollen, waren positiv für HBsAg. Anti-HBs-Test zeigen eine positive von 33 HCC-Patienten (3.0%) und keiner positiven in den Kolonkrebskontrollen, während die Lungenkrebs- und Spenderkontrollen eine Prävalenz von 11.4% und 10.7% respektiv. Keine Gruppe unterschied sich signifikant von einer anderen im Test mit Yate's correction, jedoch.

Anti-HBs kam auch in der HCC-Gruppe signifikant häufiger häufiger als in der Kontrollgruppe, wie gezeigt in Tabelle III.
HEPATITIS B AND HEPATOCELLULAR CARCINOMA

TABLE III
FREQUENCY OF ANTI-HBc IN HBsAg-NEGATIVE SERA

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Anti-HBc positive</th>
<th>p'</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC</td>
<td>29</td>
<td>12 (41.4%)</td>
<td></td>
</tr>
<tr>
<td>Colon Ca</td>
<td>38</td>
<td>2 (5.3%)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Lung Ca</td>
<td>45</td>
<td>0</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Blood donors</td>
<td>56</td>
<td>0</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

1 Determined by X^2 test with Yates's correction, control compared with HCC group. 2 One specimen was positive for both anti-HBs and anti-HBc.

The five HBsAg-positive specimens were also anti-HBc-positive, and anti-HBc occurred alone in 41.4% of the HBsAg-negative HCC sera. In the colon cancer group, anti-HBc occurred in two patients (5.3%), while in the lung cancer patients and blood donors, anti-HBc was not detected.

When HBsAg-positive sera were grouped with those positive for anti-HBc alone (i.e., those sera showing evidence of HBV infection without an anti-HBs response), this combination accounted for 48.5% of the HCC patients, 5.3% of the colon cancer patients, and none of the two other control groups (Table IV). The HCC patients differed significantly from all of the control groups in this respect (p<0.001). The combination of anti-HBs and anti-HBc in a serum was uncommon, being found only once in the HCC group. The HCC patients also differed significantly from the control groups with respect to the overall frequency of HBV infection, as reflected by positivity for any of the three markers (Table IV). The rates of infection were similar in the three control groups - 5.3%, 11.1%, and 10.6% - and significantly lower than the rate seen in the HCC group (51.5%).

Four of the 16 HCC patients tested were positive for anti-HBe. None was positive for HBeAg. All of the control sera tested were negative for both.

TABLE IV
OVERALL SEROPOSITIVITY FOR HBV INFECTION IN HCC PATIENTS AND CONTROLS

<table>
<thead>
<tr>
<th></th>
<th>HBsAg, anti-HBs or anti-HBc</th>
<th>HBsAg and/or anti-HBc only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Positive</td>
</tr>
<tr>
<td>HCC</td>
<td>33</td>
<td>17 (51.5%)</td>
</tr>
<tr>
<td>Colon Ca</td>
<td>38</td>
<td>2 (5.3%)</td>
</tr>
<tr>
<td>Lung Ca</td>
<td>45</td>
<td>5 (11.1%)</td>
</tr>
<tr>
<td>Blood donors</td>
<td>56</td>
<td>6 (10.7%)</td>
</tr>
</tbody>
</table>

1 Determined by X^2 test with Yates's correction, control compared with HCC group. 2 One specimen could not be tested for anti-HBs.

TABLE V
HBV SEROPOSITIVITY VERSUS HISTORY OF ALCOHOLISM IN HCC PATIENTS

<table>
<thead>
<tr>
<th></th>
<th>HBV positive</th>
<th>HBV negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcoholic</td>
<td>7 (87.5%)</td>
<td>1 (12.5%)</td>
</tr>
<tr>
<td>Non-alcoholic</td>
<td>9 (69.2%)</td>
<td>4 (30.8%)</td>
</tr>
</tbody>
</table>

1 Positive for HBsAg, anti-HBs or anti-HBc.

History of alcoholism
In 21 HCC patients a positive or negative history of alcoholism could be obtained by interview or from hospital records. Similar proportions of alcoholic and non-alcoholic patients showed evidence of HBV infection, and comparison by X^2 test showed no significant difference between the two groups (Table V).

Discussion
This study demonstrates a high frequency of hepatitis B virus infection in a group of hepatocellular carcinoma patients from the Philadelphia area, where both HBV infection and HCC are relatively uncommon. Moreover, the association between the two conditions is statistically significant when the HCC patients are compared with other cancer patients and with a group of healthy blood donors.

Of interest is the fact that the frequency of HBsAg positivity in our HCC patients is roughly half that found by Tabor et al. (1977) and Omata et al. (1979) in previously reported American series, while the overall prevalence of HBV infection in our series is in close agreement with the former and somewhat higher than that found by the latter. This difference between HBsAg positivity and overall HBV seropositivity is due to the many HCC patients in our series who were positive for anti-HBc alone. This category of patients was recognized in 1975 by Szmuness, who speculated that they might be individuals undergoing resolution of the chronic carrier state (Szmuness, 1975). It appears that these patients, like chronic carriers of HBsAg, have been infected with HBV but have not responded with production of the "protective" antibody, anti-HBs. Thus they may be at risk for chronic hepatitis and its possible sequelae - hepatic cirrhosis and HCC.

In a recent study of Chinese-Americans living in New York City, Szmuness demonstrated that anti-HBc generally can be detected for a longer time than HBsAg in the serum of infected patients (Szmuness et al., 1979). Other studies similarly indicate that...
anti-HBc may be the most persistent marker of HBV infection (Cohen and Cossart, 1977; Hansson, 1977; Szmuness et al., 1976). In our series of HCC patients, anti-HBc likewise appears to give a much better estimate of the prevalence of past and current HBV infection than does either HBsAg or anti-HBs.

Studies of HCC patients in which both HBsAg and anti-HBc have been assayed are limited, but they show a striking variability with respect to three parameters - HBsAg seropositivity, concurrence between HBsAg and anti-HBc seropositivity, and mean age of patients. Two studies from Senegal which show the highest frequencies of HBsAg positivity (77% and 79.4%) also show the best agreement between HBsAg and anti-HBc positivity (Blumberg et al., 1975; Larouze et al., 1976). The mean ages of the patients in these studies are 32 and 41.5 years, respectively. Our HCC patients stand at the other extreme, with a much lower frequency of HBsAg positivity (14.7%), a frequency of anti-HBc positivity three times this, and a mean age of nearly 60. Most of the other published series occupy an intermediate position with respect to these three parameters (Blumberg et al., 1975; Omata et al., 1979; Bassendine et al., 1979; Trichopoulos et al., 1978; Kubo et al., 1977), a notable exception being Omata's California series, which has a mean age similar to ours (56.7 years) but close agreement between HBsAg and anti-HBc positivity (26% and 32%) (Omata et al., 1979).

Population studies indicate that HBsAg positivity peaks in the third to fifth decades of life and declines in the sixth decade (Szmuness, 1975; Papavangelou et al., 1977). Thus, the lower frequency of HBsAg in our patients when compared with the Africans may be partly or wholly the result of an age effect, as others have suggested (Szmuness, 1978; Vogel et al., 1970). Other possible explanations, however, might include genetically or environmentally determined differences in host response to HBV infection, or even infection with different strains of the virus.

The frequency of anti-HBs in our HCC patients (3%) was lower than that in our lung cancer patients (11.1%) and donors (10.7%), although the difference was not statistically significant. Studies from HBV-endemic areas have consistently shown lower frequencies of anti-HBs in HCC patients than in controls (Blumberg et al., 1975; Larouze et al., 1976; Macnab et al., 1976; Vogel et al., 1972). One American series, however, reports the opposite, with 33% of their patients and 4% of their controls positive (Tabor et al., 1977).

Our finding of several sera positive for anti-HBe and none positive for HBeAg agrees with findings of several previous studies (Tabor et al., 1977; Werner et al., 1976; Eleftheriou et al., 1975) and is not surprising for two reasons: (1) anti-HBe has been shown to correlate with low circulating levels of HBsAg (Skinner, 1977), and low levels of antigenemia seem to be a feature of HCC patients (Szmuness, 1978); and (2) the frequency of anti-HBe in HBV-infected persons also seems to increase with age (Skinner, 1977). Since HBeAg shows a positive correlation with the production of Dane particles and anti-HBe a negative correlation, Werner's suggestion that HCC patients are unlikely to have circulating whole virus may apply to patients from non-endemic as well as endemic areas (Werner et al., 1976).

The number of HCC patients in which a history of alcohol intake was available is small, but our study seems to show similar rates of HBV infection in alcoholic and non-alcoholic patients. Thus, it agrees with the recent British study (Bassendine et al., 1979) but disagrees with two California studies which suggest a significant association between HBV infection and HCC only in non-alcoholic patients (Peters et al., 1977; Omata et al., 1979). In comparing these studies with our own, however, it is important to note that the California patients were divided into those with alcoholic or non-alcoholic liver disease largely on the basis of histopathology, while our own patients were grouped as alcoholic or non-alcoholic according to history. The California series also include many patients of Chinese and Mexican descent, while our own included only one Oriental, the remainder coming from non-endemic areas of North America and Europe.

In summary, this study has used matched control groups of cancer patients and blood donors to demonstrate a strong association between hepatitis B virus infection and hepatocellular carcinoma in our study population. We feel that this association supports the hypothesis that HBV infection plays an important role in the development of HCC in areas such as the United States as well as in areas in which both conditions are endemic. Although the exact role of HBV in the oncogenesis of HCC remains obscure, major advances in this area are likely to be forthcoming from experimental work with the newly described woodchuck hepatitis-hepatoma model (Summers et al., 1978; Werner et al., 1979) and from prospective studies of human populations. Studies of the latter type, such as that of Kubo et al. (1978) in Japan and the larger study currently being conducted by Beasley in Taiwan (Beasley and Lin, 1978), have begun to define the substantial risk of HCC for individuals chronically infected with HBV. At the same time they provide a means to observe patients at all stages of the progression from apparently uncomplicated HBV infection to HCC. These observations will almost certainly lead to valuable insights into the pathogenesis of hepatocellular carcinoma and hopefully will also suggest strategies for preventing or altering the course of this almost invariably fatal disease.

ACKNOWLEDGEMENTS

This work was supported by USPHS grants CA-06551, RR-05539, and CA-06927 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania. We thank Drs. A.R. Crane and I. Millman for their assistance, Drs. J.F. Hruska and W.T. London for their commentary, and Mrs. Margaret Aldrich and Ms. Jacqueline Adamczyk for preparation of the manuscript.
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HEPATITIS B VIRUS AND THE PREVENTION OF PRIMARY HEPATOCELULAR CARCINOMA

Primary hepatocellular carcinoma (PHC or hepatoma) is one of the most lethal and most common cancers in the world. Before 1950, several pathologists noted that PHC usually occurred in livers that were affected with cirrhosis. In the 1950s, workers in West and East Africa suggested that the cirrhosis was of the postnecrotic type, and therefore that both cirrhosis and PHC were end results of viral hepatitis. It was not possible to test this hypothesis at that time, since the virus could not be detected. After the discovery of Australia antigen and its identification in 1967 with a hepatitis virus (later designated hepatitis B virus or HBV), that hypothesis could be directly studied. When sensitive tests for the surface antigen of HBV (HBsAg) were developed, a series of studies in Africa and Asia consistently showed a much higher frequency of persistent infection with HBV in patients with PHC than in appropriate controls.1

Encouraged by those findings, we and others in Africa, Asia, Europe, and America proceeded to test the hypothesis that infection with HBV was required for the development of most cases of PHC. The results of these studies will be summarized here. After reviewing this evidence, the reader may wish to decide that it is sufficient to conclude that the hypothesis is more likely to be supported than rejected. If such a conclusion is reached, it will be appropriate to test a consequent hypothesis: that prevention of HBV infection will eventually decrease the incidence of PHC.

In 1969 Millman and Blumberg introduced a novel method for the preparation of a vaccine against HBV; the vaccine is produced by extraction of particles containing only HBsAg from the peripheral blood of HBV carriers.2 In a recent field trial, Szmuness et al.1 found that this vaccine was safe and highly effective. If their findings are supported by other trials now in progress, it is likely that the hepatitis B vaccine will become available for clinical use within the next one or two years. Along with other public-health measures, the vaccine could then be used to prevent infection with HBV in large populations and could thereby permit a direct test of the initial and derivative hypotheses.

In presenting the several bodies of data that support the hypothesis of a relation between PHC and persistent infection with HBV, the method of independent evidence is used. Although any single item may have an alternative explanation, the total body of information is best explained by the stated hypothesis.

First of all, PHC occurs commonly in regions where chronic carriers of HBV are prevalent and much less frequently in areas where they are not. Secondly, case-control studies have shown that 90 per cent or more of patients with PHC who live in areas where HBV is endemic have HBsAg or high titers of antibody against the core antigen in their blood. These markers can be considered evidence of current or previous persistent HBV infection. In the same areas, controls have markedly lower frequencies of HBsAg and antibody to HBV core antigen. Even in the United States, where PHC is uncommon, patients with the disease have higher prevalences of HBsAg and especially of antibody to core antigen than do controls. In other words, in areas of both high and low PHC incidence, serologic evidence of persistent infection with HBV is more common in patients with PHC than in controls.4

Most cases of PHC (approximately 80 per cent) arise in a liver already affected with cirrhosis or chronic active hepatitis or both. If chronic hepatitis and cirrhosis are steps toward the development of liver cancer, then case-control studies of these two diseases should also show higher prevalences of chronic infection with HBV in the cases. Studies in Africa and Korea have confirmed this prediction.4,5

HBV proteins can usually be demonstrated with histochemical stains or immunologic techniques in the hepatic tissues of patients with PHC. HBsAg and hepatitis B core antigen are undetectable or present only in small quantities in the tumor cells themselves, but are found in the nonmalignant cells adjacent to the expanding tumor and elsewhere in the liver.6 These antigens are not found in the livers of uninfected persons nor in persons with antibody to HBsAg in their serum.

If persistent HBV infection causes PHC, such infection should precede the occurrence of PHC. To test this hypothesis, it is necessary to identify asymptomatic chronic carriers of HBV and controls who are not carriers and to follow them for several years to see whether PHC develops. A major study of this type is being conducted by Beasley and Lin in male civil servants between the ages of 40 and 60 years in Taiwan.7 Approximately 3500 carriers were identified. The controls are 3000 HBsAg-negative men matched by age and place of origin in mainland China or Taiwan and approximately 16,000 other HBsAg-negative men between the ages of 40 and 60 who were not matched for place of origin. The subjects have been followed for two to four years. Fifty cases of PHC have occurred during the follow-up period, and all but one have been in chronic carriers. Thus far, the relative risk of PHC is more than 250 times greater in carriers than in non-carriers, and 98 per cent (the attributable risk) of the cases have occurred in carriers.7,8

Because PHC usually develops in a liver that is affected by cirrhosis or chronic hepatitis or both, some investigators have argued that any hepatotoxic agent that causes cirrhosis is associated with an increased risk of PHC, and that hepatitis B virus is one such agent. A rigorous test of the hypothesis that chronic infection with hepatitis B virus increases the risk of PHC in addition to producing cirrhosis is to compare the incidence of PHC in patients with cirrhosis who are or are not chronic carriers of HBV. Obata et al. have performed such a study in Japan. Seven of 30
HBsAg-positive patients with cirrhosis (23 per cent) but only five of 85 HBsAg-negative patients with cirrhosis (6 per cent) had PHC after about four years.9

In populations where HBV is endemic (sub-Saharan Africa, Asia, and Oceania), there is good evidence that many of the chronic carriers acquire HBV as a result of infection transmitted from their mothers early in life (at the time of delivery, in the period after birth when the mother and child have considerable close contact, or perhaps prenatally). That is, the mothers themselves are chronic carriers, and offspring born when the mothers are infectious are likely to become chronic carriers. Within a population, persons infected at birth or during the first year of life will have been chronic carriers of HBV longer than persons of similar age who are infected later in life. Therefore, if the duration of being a chronic carrier is related to the likelihood of having PHC, one could predict that the mothers of patients with PHC would be more likely to be chronic carriers than the mothers of controls of similar age who do not have PHC. Studies in Senegal, West Africa,10 and in Korea11 are consistent with this prediction.

Most studies of the relation of viruses to cancer have been conducted in laboratory animals or tissue-culture systems. It is thought that the genomes of DNA viruses or DNA transcripts of RNA viruses become integrated into the genomes of host cells, and that the product of a viral gene is required to produce malignant transformation of the cell. Integrated DNA sequences of HBV have been identified in a cell line derived from a primary hepatocellular carcinoma removed from the liver of an African patient.11 In addition, Brechot et al. have reported integration of DNA from HBV into the primary-tumor cells of three patients with PHC.12 In these primary tumors, the integration sites were probably identical in each cell within the tumor, suggesting that the integration event was present in the initial cell that gave rise to the tumor.

Persistent infection with a virus similar to HBV is associated with a naturally occurring primary carcinoma of the liver in Marmota monax, the woodchuck or groundhog. Robert Snyder has trapped Pennsylvania woodchucks in the wild and maintained a colony at the Philadelphia Zoological Garden for the past 20 years. Post-mortem examinations were performed in more than 100 woodchucks, and about 25 per cent of the animals had primary liver cancers. The tumors in the animals were usually associated with chronic hepatitis. Summers, a virologist at the Institute for Cancer Research, examined serum samples from these animals for evidence of infection with a virus similar to HBV. He based his investigation on the hypothesis that viruses in the same class as HBV would have a similar nucleic acid structure and similar DNA polymerase. HBV was known to have unique characteristics: it contains a circular, double-stranded DNA genome with a single-stranded region and a DNA polymerase capable of filling in the single-stranded region to make a fully double-stranded, circular DNA. Summers found that about 15 per cent of the woodchuck serum samples had particles containing a DNA polymerase and a DNA genome that were similar in size and structure to those of HBV. Examination of pellets from these serum specimens with an electron microscope showed the three types of particles associated with HBV. Later, Werner et al. showed cross-reactivity of the core and surface antigens of the virus in woodchucks (WHV) with the comparable antigens of HBV.13 A close association between persistent WHV infection and PHC has also been found; DNA from WHV hybridized to the cellular DNA in five woodchuck livers containing PHC but did not hybridize to the DNA in nine livers without tumors. Finally, Summers and his colleagues have demonstrated integration of one or two WHV genomes into tumor-cell DNA in two woodchuck primary liver cancers. Integration appeared to occur at the same unique site in each cell of the tumor. Thus, each tumor was a clone with respect to the integrated viral DNA.14

Liver cancer in the woodchuck is not what is generally regarded as a laboratory model of a human disease — that is, it was not designed or "created" by an investigator for research purposes; rather, it is a naturally occurring disease related to a naturally occurring virus, both of which have remarkable features in common with their human counterparts.

Additional viruses with similarities to HBV have been found in Chinese domestic ducks (in which there is a high frequency of liver cancer) as well as domestic Pekin ducks in the United States. They have also been found in California ground squirrels (Spermophilus beecheyi). We have termed HBV and the viruses similar to it "Icorons" — an acronym based on the name of the Institute for Cancer Research, where three of these viruses were discovered.

Our interpretation of these nine lines of evidence is that, taken together, they strongly support the hypothesis that persistent infection with HBV is required for the development of most cases of PHC, and therefore that the next step is warranted: testing of the hypothesis that decreasing the frequency of HBV infection will in due course decrease the frequency of PHC. The availability of the hepatitis B vaccine produced from HBsAg in human blood and increasing knowledge of the mechanisms of transmission of HBV will make such a study feasible. Since the incidence of cancer is high in HBV carriers, it may be possible to measure the effect of the program within a reasonable time. In any case, the control of HBV infection is clearly justified as a public-health measure for the prevention of acute and chronic hepatitis and post-necrotic cirrhosis, diseases of major importance in the same regions where PHC is common.

The associations of HBV and WHV with this carcinoma suggest that there may be other, similar virus-cancer relations that could be dealt with by primary-prevention strategies. We believe that searching for
such viruses and cancers in animals and human beings is an important new direction for cancer research.

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W. Thomas London, M.D.

References


A Cellular Model of the Role of Hepatitis B Virus in the Pathogenesis of Primary Hepatocellular Carcinoma

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We have devised a model to explain the role of hepatitis B virus (HBV) in the pathogenesis of acute and chronic liver disease and primary hepatocellular carcinoma. The model postulates that the liver contains two populations of cells—R and S cells. R cells are resistant to productive infection with HBV, whereas S cells (susceptible cells) actively replicate virus when infected. As a result, infected S cells have a shortened survival; their death stimulates the growth (regeneration) of R cells. The model also proposes that R cells are less mature than S cells. They can divide and produce R cells or divide and differentiate into S cells, but S cells can only give rise to other S cells.

Integration of viral DNA may occur in either R or S cells, but only integration of HBV DNA in R cells is of potential tumorigenic significance. R cells are already being stimulated to proliferate by the death of S cells; the site or arrangement of integrated DNA may increase the expression of one or more cellular genes providing an additional growth advantage for a particular R cell over other proliferating R cells. As this clone expands, it may develop the phenotypic characteristics of a neoplasm. A clinical tumor will not result, however, unless the stimulus to cell division is maintained by the death of S cells. Integration of HBV DNA sequences into S cells is not of tumorigenic significance. S cells have a limited capacity for cell division, and integration of viral DNA does not increase that capacity.

In this report, the model is used to explain various phenomena associated with HBV infection, and new data presented at the Kroc conference are used to test the model.

Infection with hepatitis B virus (HBV) can have many outcomes. Transient infections can result in subclinical disease, acute anicteric hepatitis, acute icteric hepatitis, or fulminant hepatitis. Persistent infections may give rise to no detectable liver disease, chronic persistent hepatitis, chronic active hepatitis, postnecrotic cirrhosis, or primary hepatocellular carcinoma (PHC). The mechanisms by which one virus can produce so many different effects in the same organ, the liver, are poorly understood. We have devised a model that provides at least a partial explanation of these and other phenomena (listed below) associated with HBV infection. The purpose of the model is to generate testable hypotheses of the role of HBV in the pathogenesis of acute and chronic liver diseases and PHC.

A pathogenetic model of HBV infection should attempt to explain the following phenomena:

(i) PHC is associated with persistent but not with transient HBV infection (1).
(ii) Persistent infection is also associated with chronic hepatitis and cirrhosis (2, 3).
(iii) Acute hepatitis is usually associated with transient rather than persistent infection (2, 3).
(iv) There is a long “incubation” period (20 to 40 years) between the onset of persistent HBV infection and the development of PHC (2, 3).
(v) Hepatitis B surface antigen (HBsAg) is commonly found in nonneoplastic cells, but only rarely in the neoplastic cells of a liver containing PHC (4).
(vi) Sequences of HBV DNA are found integrated into the genomes of both tumor and nontumor cells of livers containing PHC (5, 6).
(vii) The concentration of HBsAg in the serum of patients with PHC is low (2, 3).
(viii) In areas where PHC is endemic, the incidence of liver cancer in males is 4 to 9 times that in females (7).
(ix) Infants born to carrier mothers do not become HBsAg(+) until 2 to 3 months of age. Fetuses do not produce HBsAg (8).
(x) Infants frequently develop persistent infections...
with HBV, whereas adults usually develop transient infections (8, 9).

THE MODEL

Our model (2, 3) proposes that the liver contains two populations of hepatocytes: (a) cells that are susceptible to productive infection with HBV, hereafter called S cells; and (b) cells that are resistant (or relatively resistant) to productive infection, hereafter called R cells. Integration of HBV genomes can occur in either R or S cells. We assume that infected S cells have a shortened survival. This could occur by several mechanisms. Virus production probably compromises host-cell metabolism sufficiently to eventually kill the cell. Secondly, there may be an immune response to viral-cellular antigen complexes on the surface of hepatocytes replicating virus (10). Thirdly, infected S cells may be more vulnerable to the effects of toxic oxygen radicals than uninfected cells. [Vierucci et al. have shown that HBV proteins cause the release of superoxide anions from polymorphonuclear leukocytes (11).]

In the liver, the stimulus for cell division is the death or removal of hepatocytes (12). Hence, persistent infection with HBV leads to continuous cell death and to continuous regeneration. Infected S cells have the greatest risk of dying; R cells, on the other hand, are not productively infected and are, therefore, likely to increase in number.

Integration of viral DNA into an R cell may have tumorigenic significance. Since R cells are already being stimulated to proliferate by the death of S cells, the site or arrangement of integrated DNA and the associated rearrangements of cellular DNA may increase the expression of one or more cellular genes providing an additional growth advantage for a particular R cell over other proliferating R cells. As this clone expands, it may, by the process of clonal evolution, develop the phenotypic characteristics of a neoplasm. The point here is that integration, expression of a cellular oncogene, and phenotypic transformation are insufficient to produce a clinical tumor unless the stimulus to cell division is maintained. There must, of course, be some stage at which the R cell clone (or subclone) acquires sufficient autonomy to not require external stimuli, but we assume that this is quite late in the process.

This model requires two populations of hepatocytes, one susceptible and one relatively resistant to HBV infection. Such cell populations have not yet been identified in the human liver. However, it is known that the liver will respond to repeated cytotoxic insults with repeated rounds of regeneration eventually leading to liver failure (12). Therefore, it is likely that there are less mature cells which can divide and differentiate further, and fully differentiated cells which either cannot divide or can only divide in 1 or 2 times. We have proposed that R cells are the less mature, and S cells are the fully differentiated hepatocytes. According to this scheme, an R cell can divide and yield two R cells, or one R cell and one S cell, or two S cells. An S cell, if it can divide at all, can only produce S cells (Figure 1). Since the selective pressure of HBV infection is applied mainly to S cells, R cells will gradually accumulate (Figure 2). The model hypothesizes that integration of an HBV genome into an R cell decreases the probability of differentiation into S cells. Phenotypic transformation of an R cell ensures that it will only give rise to other R cells. The integration event may, in addition, result in increased (or decreased) expression of a cellular gene (or genes) which may provide a further growth advantage (R cell surrounded by dashed line in Figure 2). If this occurs, the clone arising from that R cell will soon outgrow all other R cells. Even at this stage, the model postulates that this clone will not become a clinically significant tumor unless growth stimulation is maintained by selective pressure against S cells. Further steps, collectively known as clonal evolution, are required before a clone or subclone can become a clinical tumor.

Integration of viral DNA and malignant transformation of an S cell would not result in a clinically significant tumor both because of the limited capacity of an S cell to divide and its susceptibility to productive infection with HBV.
EVIDENCE FOR R AND S CELLS

Is there evidence that hepatocytes differ in their susceptibility to HBV infection? Several investigators have attempted, using serial liver biopsies and immunofluorescence or immunoperoxidase techniques, to identify the patterns of HBV infection within the liver. Studies in adult humans have, in general, shown that at the time of diagnosis of acute hepatitis B [when alanine aminotransferase (ALT) levels are elevated], very few cells demonstrate either HBsAg or hepatitis B core antigen (HBcAg) (13, 14). HBsAg is present in the plasma prior to the rise of liver enzymes. Observations of the pattern of HBV infection in the liver during the incubation period of hepatitis have been made on experimentally infected chimpanzees (15-17). In these animals, liver enzymes rise 6 to 10 weeks after the appearance of HBsAg in plasma. Initially, the cell membranes of a few hepatocytes show a low-intensity staining for HBsAg in a "honey-comb"-like pattern. Cells showing this pattern of staining increase in number and are accompanied by the appearance of cells whose nuclei stain for HBcAg and less commonly other cells which demonstrate HBsAg in their cytoplasm. Very few cells have both cytoplasmic HBsAg and nuclear HBcAg. Eventually, 90% or more of hepatocytes demonstrate membrane staining of HBsAg and up to 90% nuclear staining of HBcAg; diffuse cytoplasmic staining of HBsAg is rarely seen. Cells demonstrating staining for HBV proteins begin to disappear from the liver prior to the rise in ALT and continue to disappear until very few are demonstrable at the time ALT level reaches its peak.

When a virus infects a cell, it must adsorb to the cell surface, penetrate the cell membrane and reach some specific intracellular site before viral replication can begin. Very little is known about these events with respect to HBV. One interpretation of the immunofluorescence studies, following acute infection, is that most hepatocytes have receptors for HBV, as demonstrated by HBsAg staining of cell membranes. A smaller population of cells replicate virus as shown by nuclear staining for HBcAg or cytoplasmic staining for HBsAg. Whether the rapid disappearance of nuclear- and membrane-stained cells coincident with the ALT rise indicates elimination of these cells or a combination of suppression of viral replication and cell death is unclear.

In persistent HBV infections in humans, the immunohistology is more explicit and better documented than in acute infections (18, 19). In patients with chronic persistent hepatitis or chronic carriers without histologic evidence of liver disease, hepatocytes showing diffuse cytoplasmic staining for HBsAg are scattered throughout the liver. In some areas, aggregates of HBsAg(+) cells are seen. With hematoxylin and eosin stains, the cytoplasm of these cells frequently has a "ground-glass" appearance. Precise counts of stained and unstained cells in the liver of chronic carriers have not been reported, but the impression is that 30 to 80% of cells are HBsAg(+). HBcAg is found in only a few per cent of hepatocytes in "normal" carriers, whereas in carriers receiving immuno-suppressive drugs (renal transplant patients), 50 to 90% of cells may contain core antigen.

In patients with chronic active hepatitis, HBcAg may appear in up to 60% of liver cells together with membranous or cytoplasmic HBsAg. The positive cells tend to appear in clusters. In patients with postnecrotic cirrhosis, clusters of cells are again seen showing cytoplasmic staining for HBsAg. By orcin and immunoperoxidase stains of formalin-fixed, paraffin-embedded tissue, HBsAg is seen in the form of discrete cytoplasmic bodies adjacent to, or surrounding, the nucleus. HBcAg has been detected in as many as 40% of hepatocytes, but more commonly it is identified in 5% or fewer cells (19).

In patients with PHC, HBsAg can frequently be found in the cytoplasm of some clusters of nontumor cells usually in the form of large discrete bodies. Cells showing membrane staining or multiple small granules of HBsAg in the cytoplasm are sometimes seen. The tumor cells generally do not show HBsAg. In our experience, when tumor cells contain HBsAg, it is in the small granular form. HBcAg is either not detectable or is present in less than 5% of cells (4, 19).

Collectively, these observations are consistent with the view that the majority but not all cells present in the liver of normal adults are mature hepatocytes, S cells, and that they are susceptible to infection with HBV. It is uncertain whether the membranous staining for HBsAg, without nuclear HBcAg or cytoplasmic HBsAg, seen in the early stages of acute HBV, indicates adsorption of virus particles on the cell surface or expression of viral protein in the cell membrane. In patients with persistent infection, as the liver disease progresses over time, fewer liver cells express viral protein. This is compatible with the hypothesis that S cells are selected against, and R cells accumulate.

APPLICATIONS OF THE MODEL

According to the model, the liver in the fetus and newborn would be composed mostly or entirely of R cells and would not be able to support replication of HBV (Figure 3). After birth, further division and differentiation occur giving rise to S cells which can permit viral replication. Infection with HBV during the first 6 months of life generally results in a persistent infection (Figures 3-5). Again, according to the model, infection is taking place at a time when the R cell population is decreasing and the S cell population is increasing as a result of normal processes of growth and development. Thus, as S cells become infected and gradually die, new S cells are continuously available to maintain the infection. Later in life, the reverse is true; most cells in the liver are S and few are R (Figures 3, 4). Infection may result in the death of large numbers of S cells (acute hepatitis), and too few S cells are available to maintain the infection.

The male-female difference in tumor risk is probably related to sex differences in the risk of persistent infection with HBV. Females are less likely than males to develop a persistent infection following initial exposure to HBV and more likely to develop anti-HBs. Among individuals infected for more than 1 year, females are more likely than males to eventually clear the infection. In terms of the model, selective pressure against S cells is maintained for a longer time in males than in females. One hypothesis...
Fig. 3. The proportion of R and S cells varies at different stages of life.

Fetus and Newborn
Too few S cells to sustain infection.

Infant 3 to 6 Months
Sufficient S cells for infection. R cells differentiate into S cells to maintain infection.

Adult
Most cells are S cells. Too few R cells differentiating into S cells to maintain infection.

Fig. 4. The availability of S cells at different stages of life influences the kind of infection and liver disease which develops.

Fig. 5. Chronic infection with HBV results in elimination of S cells, generation of new S cells by division and differentiation of R cells, and the gradual accumulation of R cells. R cells containing integrated HBV DNA sequences are unable to differentiate into S cells and, therefore, accumulate more rapidly than other R cells.

derived from this model is that males maintain a higher proportion of R cells in their livers throughout their lives than females. That is, the distribution of R and S cells in adult male livers is closer to the 3- to 6-month-old infant than the female adult pattern (Figures 3, 4).

TESTS OF THE MODEL

New information was presented at this conference. These data, since they were acquired after the model was formulated, can be used as informal tests of the model. The studies of Bréchot et al. (20), Shafriz (21), and Summers and Mason (22) confirmed earlier reports from their laboratories (5, 6) that integrated sequences of HBV DNA can be found in tumor and nontumor cells in the livers of HBV-infected humans or woodchuck hepatitis virus-infected woodchucks. Since their preliminary reports were used in modifying earlier versions of the model, the new results cannot be used to test it. Nevertheless, it is important to reiterate that according to the model, integration occurs in both R and S cells, but only R cells are at risk of giving rise to tumors.

The studies of Burrell et al. (23) concerning in situ hybridization of HBV DNA in human liver biopsy specimens are pertinent to the model. His method was capable of detecting cells containing 10 or more HBV genomes, suggesting that only cells actively replicating virus were identified. This was supported by the findings that, in 90% of the cells positive for hybridization, the HBV DNA sequences (grain counts) were localized to the cytoplasm and that most of the grain counts were contributed by sequences of single-stranded HBV DNA. As predicted by the model, the cells actively replicating virus (positive for HBV DNA) were focally distributed and, histologically, were undergoing degeneration. According to the model, the cells which produce virus are S cells and their survival is shortened as a result of the infection. It is the death of S cells that stimulates the growth of R cells.

A further test of the model is provided by the work of Eddleston (10). Using the patients’ own lymphocytes and hepatocytes, he found that the target antigen for HBV-infected liver cells was the core, not the surface antigen. Other studies, including those of Burrell et al. (23) presented here, suggest that the hepatocytes which express HBcAg are the ones that are actively replicating virus. That is, the host immune response is directed only against the cells actively replicating virus, not against cells with integrated HBV DNA that are not producing HBV. Again, according to the model, cells actively replicating virus and subject to immune killing are S cells and the cells not producing core antigen (not replicating virus) would be R cells.

Thus, our model can explain most of the phenomena previously associated with acute and chronic HBV infections and many of the new observations. Our research is now focused on isolating and characterizing R and S cells in woodchuck livers and testing directly the hypothesis that tumors will arise from R cells in woodchucks persistently infected with woodchuck hepatitis virus.

Acknowledgment: We wish to thank Dr. Edward Lustbader for his many contributions to our thinking about this model.
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Hepatitis B Virus and the Prevention of Primary Cancer of the Liver\(^{2,3}\)

A convincing body of evidence now exists to support the hypothesis that chronic infection with the HBV is required for the development of most cases of primary cancer of the liver in humans. The plasma-derived hepatitis B vaccine, which is now available, has in many field trials been found to be highly effective and very safe. Thus, in time, there should be a marked reduction or possibly a total elimination of primary cancer of the liver (PHC).

In this editorial we will review the evidence in favor of the hypothesis that persistent HBV infection is a necessary “cause” for very many cases of PHC. This does not rule out the possibility that other environmental factors (e.g., aflatoxins) and host factors may also play a significant role in its pathogenesis. We will then discuss several forms of prevention, the first two currently in use and a third possibly applicable in the future. [These features are detailed in (1, 2).] The forms of prevention include the following: 1) primary prevention—prevention of PHC by preventing infection with HBV, 2) secondary prevention—“prevention” of PHC in people who are already HBV-infected by detection of very early tumors and removal of them surgically, and 3) prevention by delay. We have introduced this term to describe possible ways in which asymptomatic carriers of HBV could be managed so that the onset of symptomatic chronic liver disease and PHC might be delayed for a sufficiently long period to enable the carrier to live out his or her normal life-span without experiencing many of the detrimental effects of hepatitis B infection, including cancer.

In the 1950's, Payet et al. (3), Steiner and Davies (4), and other investigators in Africa suggested that viral hepatitis might be the cause of primary cancer of the liver, on the basis of clinical, pathologic, and observational evidence. At that time it was not possible for this hypothesis to be tested directly; but after 1967, with the introduction of better methods for the detection of HBV (5), tests of this notion were undertaken.

In 1971, at a meeting on cancer in Africa held in Uganda, several studies showing a striking association between HBV and PHC were presented. This event appeared to be a turning point in the development of interest in this relationship, and the intensity of the work in the field increased. In 1975 we said (6):

> During recent years, there have been parallel developments in understanding, on the one hand, the pathogenesis of primary hepatic carcinoma ... and, on the other, the biology of Australia antigen ... and the infectious agent, hepatitis B virus (HBV), to which it is intimately related. Recently, the paths of these developments have begun to converge, and from this it is possible to design a preliminary strategy which could, if the interpretations of these data are correct, result in the prevention of many, and perhaps most, cases of one of the most widespread and deadly cancers in humans.

The evidence that was then available and that supported the statements was presented. We concluded by discussing the vaccine we had introduced (7) and possible prevention strategies. In the relatively short time since that article appeared, prevention programs have begun in Alaska (among Native Americans), Korea, Senegal, northern Italy, Taiwan, Japan, and China and among east Asians living in Los Angeles and Philadelphia. There is considerable hope that the objectives may be achieved.

**PERSISTENT INFECTION WITH HBV REQUIRED FOR THE DEVELOPMENT OF MOST CASES OF PHC**

Carriers of HBV are common in the parts of the world where PHC is most common, including such surprising locations as Alaska where HBV and PHC are common in the Eskimo population (8). A large series of case-control studies, in which the frequency of the virus in patients with PHC was compared to the frequency in controls, has been completed [reviewed in (1, 2)]. In some of these studies, which utilized the most sensitive methods for the...
detection of the virus, the frequency of infection in the cases approached 100%.

The virus can be identified by immunohistologic methods in the liver tissue of people with PHC (9). An interesting feature of this observation, shown by Popper and others (10) who have pioneered this work, is that the virus is most abundant in the cells that do not appear to have undergone malignant transformation. The neoplastic cells themselves often have no or sparse evidence of viral protein (10).

Currently, one of the most exciting areas of research is the molecular biology of HBV. The entire base sequence of the virus has been determined and confirmed by several groups (11, 12). HBV DNA is integrated into the DNA of the host liver cells in a very large percentage of PHC cases—in some studies, all the cases. However, there is also integration of HBV DNA in the liver cells of patients without PHC, including those with chronic liver disease and asymptomatic carriers of HBV. Hence, integration itself cannot explain the pathogenesis of the cancer. The site of integration of HBV DNA appears to be the same in every cell in an individual tumor (i.e., clonal), but the insertion locations of viral DNA are not the same in different tumors (13-17).

The most impressive evidence supporting the HBV-induced PHC hypothesis is epidemiologic. In one of the most convincing studies on cancer etiology, Beasley and his co-workers (18, 19) in Taiwan are studying prospectively 22,707 male government workers between the ages of 40 and 59 years. Recruitment into the study population began in November of 1975 and was concluded in June of 1978. Of the total, 3,435 men were asymptomatic carriers of HBV and the remainder were not. As of December 31, 1983, 116 cases of PHC had developed in the study population and 113 of these were in HBV carriers. Even the three tumors in noncarriers occurred in persons who showed evidence of prior HBV infection. The annual incidence for the whole population was 82.4/100,000, but for the carrier group it was 527.7/100,000 (18, 19). Beasley and Hwang (19) estimated the relative risk for the carrier men to be 217 times that of the noncarriers. The relative risk showed little variation from year to year and no secular trend. This is probably the highest odds ratio for any known environmental cause for a common cancer. On the basis of life table projections, they estimated the lifetime risk of death from PHC or cirrhosis for a Chinese male carrier to be between 40 and 50%, a remarkably high value.

Beasley's study established that the association between HBV and PHC was closer than that for any other virus and a site-specific cancer, probably for any environmental factor and a specific cancer. It has been shown also that chronic infection with the virus, rather than cirrhosis, carries the risk for cancer. Patients with cirrhosis due to causes other than HBV have a low risk for PHC, while those with cirrhosis due to HBV have a much higher risk (19, 20).

Prospective studies similar to Beasley's are now in progress with Eskimos in Alaska, with Chinese in Hong Kong and Singapore, and with Japanese in Tokyo. Early reports confirm Beasley's findings, i.e., a greatly increased risk of PHC in the carriers. In 1971 (21) we had proposed, on the basis of unusual population distribution and clinical and physical characteristics of HBV, that it represented the first identified member of a new group of infectious agents. We termed these "Icrons." Using his knowledge of the molecular characteristics of HBV, Summers (22), working at the Institute for Cancer Research, Philadelphia, PA, was searching for viruses similar to HBV. He subsequently discovered two of these (in woodchucks and ducks), and another has been identified in California ground squirrels (22).

The finding of the WHV provided another convincing piece of evidence for the hypothesis that HBV and similar viruses cause cancer of the liver. Woodchucks (Marmota monax), which develop persistent infection with WHV, predictably (>80%) develop PHC, whereas uninfected animals or transiently infected animals never or rarely develop tumors (22). Another example is seen in ducks found in China. The DHBV is similar to HBV and WHV and it is also associated with primary cancer of the liver, at least in China (23, 24).

It has been shown both indirectly and directly that many of the individuals who go on to develop PHC are infected at a very young age (25). In some parts of the world, particularly Asia, the transmission may be maternal. In other locations, transmission is more likely to be horizontal. In either situation, since infection at an early age increases the probability of persistent infection, protection by vaccination in infancy appears to be indicated.

VACCINE

Infectious agents may produce many antibodies in the infected host. Some of these antibodies protect against subsequent infection and others do not; production of an appropriate vaccine requires the identification of the protective antibody. At the time of the very first observations on what was then called Australia antigen, it was obvious that the virus and the antibody against its surface antigen (anti-HBs) rarely occurred in the same serum. These observations were further supported by the early studies of Okochi and his colleagues (26), who were able to demonstrate in their first transfusion studies that the patients who had anti-HBs had a lower risk of infection with HBV than those who did not have the antibody.

These observations, scanty as they were, were sufficient to stimulate the development of a vaccine. In October 1969, Blumberg and Millman (7) and Millman (27) described the methods for extracting surface antigen from the blood of carriers and its use as the source of the vaccine. This vaccine was based on the then unique concept that the viral protein could be extracted directly from human plasma by methods that would make it safe for inoculation in humans and would preserve its antigenicity (27).

Experiments in humans were not undertaken at the time of its initial proposal, and the value of this kind of vaccine was questioned by some authorities. Later (in
1970), Krugman and his colleagues (28) inoculated children with a preparation of HBV-positive serum that had been boiled for 1 minute. In challenge experiments the heated serum provided protection against HBV infection (28). This experiment had the effect of convincing manufacturers and researchers that the Blumberg-Millman vaccine would probably work, and large batches of the vaccine were prepared for use.

In an elegantly designed and executed field trial, the late Wolf Szmuness and his co-workers (29) demonstrated the safety and effectiveness of the vaccine. He used as his study population a group of male homosexuals in New York City at high risk for HBV infection (29). There have subsequently been several other field trials that confirm the effectiveness of the vaccine. It has gained the approval of the Food and Drug Administration in the United States, as well as that of licensing authorities in other jurisdictions. It is probable that about 2 million doses of plasma-derived HBV vaccines have now been administered and there have not, to our knowledge, been any significant adverse effects associated with their use.

PRIMARY PREVENTION OF PHC

Prevention programs will be based primarily, but not exclusively, on the use of the vaccine. The vaccine that is currently used is derived from plasma. This vaccine may, in due course, be replaced by vaccine produced by recombinant DNA techniques or by synthetic antigens. The price for the vaccine is currently very high, even for countries with large public health budgets. It is likely that the price of the vaccine will decrease as more is required, as more companies and countries begin to manufacture the product, and as new methods of manufacture are developed. This has already happened in South Korea, where a plasma-derived vaccine is being produced for about one-third the cost of the American or French vaccines (Kim CY: Personal communication). The duration of the efficacy of the vaccine is also not known. In the United States the current recommendations are for revaccination after 5 years. As more experience is gained, it will be possible to design more accurately the timing of revaccinations if they are required. Protection at an early age is most important since it is during this period that infection is most likely to result in the development of the carrier state. Infection in later life is more likely to lead to development of antibody against the virus and, therefore, to less risk of chronic liver disease and PHC.

Primary prevention programs can be classified in three categories.

1) Populations with low prevalence of HBV infection.—Northern European countries, most of North America, and other nontropical or subtropical areas fit into this category. Vaccination is recommended in these regions for people in high-risk groups. These include health care workers (particularly those with frequent exposure to human blood), travelers to endemic areas, military personnel, male homosexuals, individuals working on renal dialysis units, possibly dialysis patients, drug abusers, and other persons frequently exposed to HBV carriers.

Despite the demonstrated safety of the vaccine, there has been a development that has affected its acceptance. Since 1980, AIDS has been identified in certain susceptible populations, primarily in the United States. Epidemiologic evidence indicates that AIDS can be transmitted by blood transfusion, plasma products (factor VIII), or contaminated needles. Although the vaccine manufacturing process is lethal to all known viruses, some individuals have avoided immunization with the vaccine because of fear of contracting AIDS. There is no evidence that this can occur. Recently, the probable etiologic agent of AIDS has been identified as a retrovirus. The process of hepatitis B vaccine manufacture has been shown to inactivate this virus.

2) Populations of moderate prevalence in which maternal transmission is important.—In Japan, the frequency of carriers is about 3%. Evidence indicates that 30-50% of the carriers occurs as a consequence of maternal transmission. Dr. Nishioka and his co-workers in Toyko (20), among others, have been screening expectant mothers for HBsAg for several years. Those women identified as positive for HBsAg are then screened for the presence of HBcAg. For those found to be HBcAg(+), immunization of the newborn is recommended. The infant is given high-titer anti-HBsAg at birth, a second dose of anti-HBsAg is given at about 2 months of age, and vaccination is started at about 3 months of age. Several studies show that the vaccine is very effective in newborns. The vaccination programs can be started at birth or within 3 months of age, when other pediatric vaccines are administered.

In the future, more extensive vaccination programs may be used in these countries to eliminate the remaining horizontally transmitted infections. These programs could include all newborns as well as adults who are still susceptible. The extension of the program to these other groups will probably depend on the availability and cost of the vaccine.

3) Populations with a high frequency of HBV carriers.—The frequency of HBV carriers in these populations ranges from 5 to 15%. These populations include much of China, other regions in Asia, and sub-Saharan Africa. Although maternal transmission occurs commonly, particularly in Asia, it may be relatively less important than transmission from virus carriers among siblings, other family members, and members of the general community. In these populations, universal childhood vaccination beginning at birth or within the first 3 months of age has been proposed and is being used in some regions (e.g., South Korea). It is believed that public health authorities in the People’s Republic of China have also decided on a program to vaccinate all newborns. If vaccine becomes more available and less expensive, then susceptible adults may also be vaccinated.

An interesting problem arises in those low-frequency areas for HBV carriers that contain large populations from high-frequency areas of HBV carriers. For example, in the United States (particularly in California) there are
many immigrants from regions in Asia where HBV is endemic. In Philadelphia, we are testing pregnant women in this population for the presence of HBsAg. An immunization program for the newborns of the mothers positive for this antigen is now in progress. In due course, vaccination may also be made available to all newborns and to susceptible adults in these populations.

Similarly, the Eskimo and Indian populations of Alaska have a high frequency of HBV carriers and an increased risk of developing PHC (30). A program has been proposed to immunize all susceptible individuals in this relatively small population (≈60,000) as well as all newborns (Rhoades E: Personal communication).

Extensive immunization programs, therefore, are in progress already, and more are planned. In addition to the vaccine, however, there may be other measures that can be used to control hepatitis B transmission. General improvement in sanitation may have a beneficial effect. There is substantial evidence that insects, particularly bedbugs, are heavily infected with HBV (31-34). Possibly, insect control could contribute to decreasing HBV infection. Also, some human behaviors, particularly those between mothers and newborn children, could influence transmission (35).

We can now consider what can be done for the approximately 210 million people in the world who are already carriers of HBV.

SECONDARY PREVENTION OF PHC

If carriers of HBV are at a high risk of developing PHC, is there some way of detecting cancer at an early stage? Small resectable tumors can be found by following HBV carriers prospectively with periodic testing of AFP and possibly other fetal proteins (a-glutamyl transferase, acidic ferritin). Sun, as well as the Coordinating Group for Research on Liver Cancer in China, has had extensive experience using mass surveys for elevations of AFP (36). Persons with elevated AFP are investigated with imaging procedures (particularly ultrasound) which can localize small masses. If a tumor is visualized, surgery is recommended. Using these methods, Tang's group (37), at the Shanghai First Medical College, reported a significant improvement in survival. In some cases, more than 1 tumor was detected by continued surveillance of AFP levels in patients who had already undergone hepatic resection. Similar studies are being conducted in Eskimo populations in Alaska and persons of Asian origin residing in Los Angeles and Philadelphia. In Alaska at least 4 early cancers have been detected in HBV carriers monitored for AFP elevations [(38); Heyward W: Personal communication].

During the course of the next few years the value of this approach can be appraised as the results from different centers become available. The question of screening of carriers for AFP in low-frequency HBV areas is still at issue. Studies now in progress may allow the identification of a subgroup of carriers who are at particularly high risk of developing tumors.

In collaboration with Beasley in Taiwan, we are currently evaluating the role of the iron-binding proteins ferritin and transferrin in predicting the development of liver cancer in HBV carriers. A series of predictors may eventually be identified that could focus on those subgroups of HBV carriers at highest risk of PHC (39).

MODEL FOR PATHOGENESIS OF PHC

There are certain features of the HBV-PHC relationship that require an explanation not provided by conventional theory. We have introduced a model to explain these findings (40). The observations used to generate the model are reviewed and the model itself is described. New studies are now in progress to test the model, but these will not be considered here.

PHC is associated with chronic infection with HBV, not with an acute, transient infection. PHC usually occurs in a liver affected with chronic hepatitis and/or cirrhosis. Hepatitis A virus infections, which do not have a chronic form, are not associated with liver cancer. Most people who develop the carrier state of HBV and, subsequently, PHC do not experience an antecedent, acute disease. The "incubation period" for the development of PHC appears to be very long; people who develop PHC may be infected as early as the first few months of life, but the disease usually does not appear until very much later, i.e., the fifth or sixth decade. Hence, any model has to take into account the very long period of exposure of liver cells to HBV. A simple mutational model does not seem to explain all the observed phenomena.

Histologic studies of the liver tissues of patients with PHC reveal that the neoplastic cells do not contain large amounts of HBsAg, hepatitis B core antigen, or whole virus, while the surrounding cells often contain large quantities of viral components (41, 42). The amount of HBsAg in the serum of patients with PHC is low compared to that in healthy HBV carriers and individuals with other forms of chronic HBV infection. Further, the amount of HBsAg in the serum decreases as the cancer progresses.

From the molecular studies HBV DNA is known to integrate into the genome of tumor cells (14-17). HBV may also be integrated into the DNA of cells that are not transformed or in the hepatocytes of individuals with chronic infections who do not have cancer (14, 17). The integrations in nontumor tissue often appear to be clonal. Finally, chronic infection with HBV causes not only PHC but also various forms of chronic liver disease; therefore, the model must explain how the same agent can cause more than one disease.

Our model postulates that the livers of mature humans [and animals that can be infected with a similar virus (22)] contain at least two populations of cells. The first and larger component readily becomes infected with HBV, synthesizes viral proteins, and can produce infectious virus. We have referred to this population of cells as "susceptible" or S-cells.

The second and, in adults, smaller population of cells is relatively resistant to replicative infection with HBV.
We have called this group of cells “resistant” or R-cells. If these cells become infected, integration of viral DNA may occur, but they produce little or no intact virus.

In individuals chronically infected with HBV, S-cells have a shortened survival. This fact may be due to an immune response to viral and cellular antigens on the surface of the virus-infected hepatocytes, or it may be that cells that are actively replicating virus are metabolically compromised. The death of S-cells, which in a chronically infected liver would be continuous, stimulates the division of R-cells. Hence there is a continuous process of death, regeneration, and, possibly, scarring of the liver. It is this continuous process that leads to the picture of chronic liver disease and posthepatic cirrhosis, which is often associated with chronic HBV infection.

The R-cells are relatively immune to death from HBV infection, since HBV replication does not occur, and host immune responses are not actively evoked; hence, R-cells are at a selective advantage compared to the S-cells. As a result, the S-cell population gradually decreases and the R-cell population increases.

Eventually, integration of HBV DNA into the DNA of the host cells may occur. If integration occurs in an S-cell, this probably has little consequence since the S-cell will eventually die as a result of the HBV replication and the host response. If the integration occurs in an R-cell, it may lead to a tumor. The R-cell population, because of the continuing death of S-cells, is continually expanding. Integration of viral DNA may give a further advantage to a clone of R-cells originating from a cell into which HBV DNA has been integrated. The integration of the HBV DNA into the R-cell may arrest its maturation and, thus, when it divides it will produce only other R-cells. Integration of the DNA may also stimulate growth factors or oncogenes in the particular clone of cells, which will further increase growth potential. As the clone expands, it may, by the process of clonal evolution, develop the characteristics of a malignant neoplasm.

What are the origins of the R- and S-cells? We have proposed that R-cells are less mature cells and S-cells are fully differentiated hepatocytes. The R-cells are the predominant cell form in early fetal life, decreasing in number but never totally disappearing in the maturing liver. S-cells are the predominant form in the normal mature liver.

According to this scheme, under normal circumstances, a dividing R-cell will give rise to 1 R- and 1 S-cell. Under abnormal circumstances an R-cell can divide and yield 2 R-cells or it can divide and differentiate into 2 mature S-cells. The more mature S-cell, if it can divide at all, will produce only other S-cells. Since the selective pressure of HBV infection is applied mainly to S-cells, R-cells will gradually accumulate. As already noted, integration of HBV DNA into the R-cell genome could hinder further differentiation and ensure that only R-cells are produced. This will further increase the rate of expansion of the R-cell component. If the integration of DNA results in the turning on of a cellular gene, which alters the cell phenotype in some way that provides a growth advantage, the resulting clone will have an even greater growth and selective advantage. In due course this effect becomes a clinical cancer perceptible to the host.

This model can also be used to explain certain features of liver disease other than PHC. Fulminant hepatitis may occur when livers with no or few R-cells become infected. If all the S-cells are killed by replicating HBV, there would be no resistant R-cells from which new liver cells could be regenerated. This consequence would suggest some unusual therapeutic measures.

Recent studies have shown that steroid therapy may be effective for CAH not associated with HBV but may be detrimental for CAH associated with HBV infection. In terms of the model, the steroids would depress the immune response by affecting lymphocytes. This event would lead to a temporary reduction in the destruction of the hepatocytes. However, the steroids would prolong the life of the infected S-cells, encourage the chronicity of infection, and thereby impede the rate of recovery.

The model could also explain the observation that the offspring of carrier mothers who are also HBeAg(+) rarely, if ever, have evidence of infection until about 3 months of age, after which they are highly likely to become persistently infected. Newborns would have relatively few S-cells and therefore would not produce sufficient quantities of virus to be detected by current means. By 6 weeks to 3 months of age a sufficient number of differentiated (S) cells would be available to synthesize enough HBV for detection by conventional assays.

Young people rather than adults are more likely to become chronically infected. In terms of the model this likelihood could be explained by the relative abundance of R-cells in the child. When S-cells are killed by the reaction to the HBV infection, sufficient R-cells are present to provide a new source of S-cells. In the adult, however, there is a relative scarcity of R-cells. When S-cells are killed by the infection, insufficient numbers of R-cells exist to provide S-cells at a high enough rate to maintain the infection.

**PREVENTION BY DELAY**

There are some interesting features of this model that can be used to discuss what we have termed “prevention by delay.” It should be clear that this form of prevention is at present speculative.

The model posits that the driving force in the growth of the cancer is the death of S-cells as the result of replication of HBV. Hence, if we could devise methods to stop or even slow down this process, it might be possible to prevent the development of serious liver disease during the normal life-span of the individual. If this intervention could be started before a large component of R-cells had accumulated, then very mild procedures could be used over a long period rather than severe treatments over a relatively short time, such as are generally used in cancer therapy.

Most forms of cancer therapy are directed toward the killing of as many cancer cells as possible. In the process, because cancer cells and normal (particularly developing) cells have much in common, many normal cells are also...
affected. This places a limit on the aggressiveness of cancer therapy. The procedures recommended by this model are directed toward preserving the health of the S-cells rather than promoting the destruction of the infected R-cells.

The suggested "therapies" have a limited goal. It is not necessary to eliminate all the R-cells but only to keep their numbers at a sufficiently low level as to be imperceptible to the HBV carrier. Further, it is not even necessary to eliminate completely the HBV infection, which may be very difficult if integration has occurred. What is required is to slow down the process to such an extent that the presence of the virus is not perceived by the patient, that is, to prolong the asymptomatic phase of the carrier state while at the same time to attempt to decrease the risk of the HBV carrier developing chronic hepatitis and PHC.

Another aspect of this form of prevention is that it can be applied very early, although at present it is hard to say how early. It would be helpful to identify those HBV carriers who will remain persistently infected from infancy to adulthood. Methods for doing this should be uncovered with further research. Again, what is proposed is prevention rather than therapy since we hope to be able to develop a benign form of management that could be used by HBV carriers who are not sick.

What can be done to slow down the process of cancer and liver disease pathogenesis? The answer to this is not known now, but there are investigations in our laboratory and others that could have a bearing on these procedures. The goals would be to decrease the replication of HBV and to prevent the entry of newly synthesized virus into uninfected liver cells. These goals should be achieved at an early stage before R-cells accumulate significantly and before integration leading to rapid division of the R-cells commences.

Evidence exists that liver cells with high concentrations of the iron-binding protein ferritin, as well as possibly high levels of iron, are more likely to become infected and to replicate HBV. We are trying to understand this process sufficiently well to see if some management of iron or iron-binding proteins can favorably alter this process (39, 43).

A variety of chemicals, many derived from plants, exist of implementation.

A committee of the World Health Organization has recommended such a program.

Infection with HBV is costly in terms of both morbidity and mortality from PHC and chronic liver disease. HBV infection is not, however, the most important medical problem in those regions of the world where it is common; bacterial and viral diseases, malaria, schistosomiasis, filariasis, malnutrition, and other diseases also exert a terrible toll. It would be unfortunate if an anti-HBV campaign detracted from useful work on the control of these diseases. However, although HBV infection may not be the most important disease in Africa, it may well be the most important disease for which control appears feasible. This circumstance argues for the rapid implementation of a vaccination program. Hepatitis vaccination could possibly be combined with other pediatric vaccinations, thereby decreasing the effort of implementation.

For heuristic reasons, we have considered the R–S (cells) developmental model only in terms of HBV and PHC. In time, however, its application to other forms of cancer may be appropriate. The model recommends, we believe, a new and encouraging approach to cancer control in which individuals at high liver cancer risk can be detected. Prevention programs directed toward supporting the health of infected cells, rather than the destruction of malignantly transformed cells, could then be instituted.

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HEPATITIS B VIRUS
Hepatitis B Infection and Kidney-Graft Survival

To the Editor: Since our publication on the relation of hepatitis B infection before transplantation to survival of HLA nonidentical kidney grafts, two additional reports have appeared: Cuthbert et al. found that graft survival was better in anti-HBs (+) than anti-HBs (−) patients; and Rashid et al. reported results similar to ours — i.e., that early graft rejection was more common in anti-HBs (+) than anti-HBs (−) recipients. These differences are significant in all three populations (P < 0.02), although Rashid’s observations and ours differ significantly from those of Cuthbert et al. Thus, there is probably a biologic significance in the differences in behavior of the three patient populations.

One difference between the populations is in the prevalence of hepatitis B infection. The high frequencies of HBsAg and anti-HBs in Rashid’s and our populations (33.3 and 42.7 per cent, respectively) reflect the regular exposure of these patients to hepatitis B virus (HBV) in hemodialysis units. Cuthbert’s patients (11.2 per cent had HBsAg or anti-HBs) were not as heavily exposed to HBV infection in dialysis units (Ware A: personal communication), and anti-HBs probably developed as a result of prior exposures to HBV in the outside community. The prevalences of anti-HBs and HBsAg observed by Cuthbert et al. are similar to those seen in general population surveys in the United States and lower than those reported in many hemodialysis units.

Recent and frequent exposures to HBV may stimulate lymphocyte and macrophage responses to HBV antigens. If lymphocytes stimulated by HBV antigens also recognize an antigenic determinant on donor cells, early rejection may result. Elsewhere, we have suggested that there is a male-associated antigen that cross-reacts with an antigenic determinant on HBV. If anti-HBs were present as a result of past exposures to HBV cellular immune responses might not be stimulated at the time of transplantation, and responses to kidney grafts might not be affected. Hence, the conflicting results of the three transplant studies suggest that the setting and proximity of the HBV infection are important for evaluation of the correlation of anti-HBs with cellular responses to HBV and to kidney-graft survival.

Furthermore, in the clinic that we studied, as is usual in United States and European dialysis units, HBV infections were predominantly with "ay" subtypes of HBsAg whereas most infections in the outside community are with HBsAg/ad. Presentation with the y determinant may affect the subsequent response to kidney grafts.

We are indebted to Cuthbert et al. for reporting their observations. As a result, we have modified our initial interpretations and present our data with new hypotheses. Testing of these will, we hope, enlarge our understanding of the relation of responses to HBV infection with kidney-graft survival.

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TRACHEOSTOMY FOR ACUTE EPIGLOTTITIS

To the Editor: In the "Weekly Clinicopathological Exercises" published on October 20, 1977, a very interesting 34-year-old man who died of acute epiglottitis was discussed. Dr. M. Stuart Strong made the correct clinical diagnosis, and in his discussion, he stated, "Epiglottitis is not rare; indeed, it is relatively common in children, with cases encountered once or twice a week in busy pediatric units." He then pointed out that in a series of 97 cases of epiglottitis that we reported, 11 (or 12 per cent) of the patients were adults. He then goes on to state, "The disorder is not as threatening to adults as it is to children because the adult structures are more rigid." He further states, "Not all patients require intubation or tracheostomy....but most children have to be intubated, and if the circumstances for managing the intubated child are not excellent tracheostomy may be required."

I disagree with this statement and I think that there is ample evidence to support my position. We stated in our report that six of the 11 adults in our study required tracheostomy to survive. In another report on epiglottitis in adults all three patients with this disorder required tracheostomy; two died during the procedure. This report reviewed 37 other cases of acute epiglottitis in adults. When these references are individually examined, the statement that this disorder is not as threatening to adults as it is to children is not borne out. One study cited in the review described three adults with acute epiglottitis; one who survived after tracheostomy and two who died while the procedure was being performed to relieve abrupt, complete airway obstruction. Indeed, it appears that as in our study, adults require more frequent tracheostomal intubation or tracheostomy as often as children do to survive.

An earlier CPC in the Journal also emphasizes this point. In a 66-year-old woman with acute epiglottitis onset of complete airway obstruction was so rapid that one of the discussants commented, "It took approximately fifteen minutes to perform the tracheostomy after we had attempted to pass an endotracheal tube. She became progressively hypoxic during the procedure and died before the tracheostomy was completed." This is another example of the deadly, rapid and unpredictable course that this infection may pursue in the adult.

Pediatricians are now in general agreement that to prevent such catastrophes, an assured airway must be established as soon as the diagnosis of acute epiglottitis is made. As shown in our study, approximately half the 97 patients required tracheostomy to survive, and in half these cases, obstruction was so abrupt in onset that two patients died while the procedure was being attempted and one was left with decerebrate anoxic brain damage. "Prophylactic" nasotracheal intubation is now generally preferred over tracheostomy, but since it is not always successful it should not be undertaken without adequate provisions for immediate surgical intervention. Thus, it appears that adults as well as children are at high risk of abrupt complete airway obstruction with this disease. An assured airway should be established immediately as soon as the diagnosis is made in all age groups with this life-threatening infection.
Le lien entre ce qui sera appelé par la suite antigène Australia et l'hépatite, a été découvert à la fin de 1966; l'hypothèse d'une relation entre l'antigène, les particules de 20-24 nm qui lui sont associées et le virus de l'hépatite, a été formulée peu de temps après (1). En 1968, nous avons été informés par le gouvernement fédéral des États-Unis d'Amérique, qui nous allouait une grande partie de nos crédits, qu'il souhaiterait voir naitre des applications de cette recherche fondamentale qu'il subventionnait depuis si longtemps. C'est ainsi que nous avons eu la possibilité de produire un vaccin contre le virus de l'hépatite par une nouvelle méthode.

Des observations faites pendant de nombreuses années et des faits plus récents (2, 3) suggèrent que l'infection par le virus de l'hépatite confère une immunité prolongée, conséquence probable de l'apparition d'anticorps protecteurs. Si l'antigène Australia est une partie du virus de l'hépatite, nous avons pensé, les anticorps spécifiques dirigés contre lui pourraient être protecteurs. De plus, l'antigène peut être obtenu simplement à partir du sang périphérique des porteurs asymptomatiques de l'antigène Australia, qui ont des taux très élevés de cet antigène. Beaucoup de vaccins anti-viraux sont produits à partir de virus développés en culture de tissu ou sur œuf embryonnée; on produit ainsi des vaccins vivants, atténués ou tués; mais il n'a pas été possible de cultiver le virus de l'hépatite B de cette manière. En outre, la source représentée par le sang humain pourrait bien avoir réellement quelques avantages, comparée aux cultures de tissus, onéreuses et biologiquement complexes.

Plusieurs arguments rendaient peu vraisemblables que les petites particules (20-24 nm) représentaient le virus entier. En 1967, nous avons trouvé que le sérum des mongoliens présentant une hépatite anicterique, contenait des concentrations de HBs Ag à des taux aussi forts que $10^{12}$ particules par millilitre de sérum. Si ces particules avaient représenté le virus entier, il paraissait inconcevable qu'une telle infestation soit compatible avec la survie des patients! Nos premiers travaux biochimiques et physiques (4, 5) s'accordaient avec le fait que l'antigène Australie (appelé antigène de surface de l'hépatite B, en abréviation HBs Ag) était probablement l'enveloppe du virus. La fraction purifiée, qui contient l'HBs Ag, comprend les particules de 20-24 nm ayant une densité en chlorure de césium de 1,20-1,21. Les traces d'acide nucléique, détectées dans ces particules, furent considérées comme insuffisantes pour coder un virus dont le poids moléculaire est estimé à $3 \times 10^{6}$. Les particules contenaient des lipides et des glycoprotéines, les lipides étant probablement responsables de la faible constante de sédimentation.

Les expériences préliminaires indiquaient que l'HBs Ag du sang des porteurs humains pouvait être purifié, de sorte qu'il soit grossièrement dépourvu d'acide nucléique et des composants du sérum humain normal. Nous avons admis que l'absence d'acide nucléique était la meilleure indication de l'absence de virus complet infectant. Nos précédentes études sur la transmission expérimentale de l'antigène Australie à des singes nouveau-nés (des vervets africains) étaient compatibles avec cette hypothèse. Des préparations partiellement purifiées pouvaient être transmises et repliquées, mais les préparations les plus purifiées (dans lesquelles les acides nucléiques sont probablement éliminés) ne l'étaient pas. En octobre 1969, travaillant pour l'« Institute for
Cancer Research » (ICR), nous avons déposé une demande de brevet pour un vaccin préparé à partir de l’antigène Australia et pour un procédé de fabrication. Ce brevet a été pris successivement pour les USA (janvier 1972) et pour d’autres pays (7).

Il y a maintenant plusieurs séries d’études utilisant des vaccins et des matériaux vaccinants contenant l’HBs Ag (antigène Australia), préparés par différentes méthodes. En 1970, Krugman rapporte des expériences faites avec du sérum non purifié contenant l’HBs Ag et inactivé par la chaleur (8). Une préparation, le MS-2, qui avait été utilisée dans des expériences antérieures, était diluée dans de l’eau distillée et chauffée à 98 °C pendant une minute. A la suite de cela, Krugman annonça que la chaleur détruisait l’infectiosité, tout en laissant persister l’antigénicité : 29 enfants d’une institution pour handicapés mentaux, n’ayant jamais eu de contact avec le virus B, ont été inoculés avec la préparation MS-2 traitée par la chaleur. Environ 1 enfant sur 2 reçut 3 injections du vaccin inactivé. 4 à 8 mois après la dernière injection, ils étaient inoculés avec le sérum non chauffé contenant le virus actif. Krugman indiqua que, chez 20 (69 p. 100) des 29 enfants, l’hépatite B a été prévenue ou modifiée dans son expression clinique. La maladie était atténuée chez les 9 autres enfants et était associée à une réduction de l’incidence de l’état de porteur chronique. En juin 1971, Soulier, lors d’un séminaire qui s’est tenu au Centre international de l’Enfance à Paris, a publié que le sérum chauffé, contenant le virus de l’hépatite B, peut être utilisé pour protéger contre l’infection. Soulier chauffa sa préparation pendant 10 heures, à 60 °C (9). Bien que les travaux de Krugman et de Soulier confirmèrent l’efficacité du vaccin, leurs protocoles expérimentaux pêchaient sur plusieurs points importants.

C’est à peu près à cette époque, que des études expérimentales sur l’animal ont été entreprises. Nous avons tout d’abord tenté d’identifier un animal d’expérience qui serait satisfaisant pour des études sur la transmission ; nous avons testé l’antigène Australia chez de nombreuses espèces : animaux de laboratoire, pensionnaires de zoo et animaux domestiques, afin de déterminer quelles espèces pouvaient être naturellement infectées par le virus de l’hépatite B. Nous pensions que les espèces naturellement infectées seraient les meilleurs sujets d’expérience. Nous avons trouvé que, seulement certains primates (chimpanzés, gibbons, vervets verts d’Afrique, etc.) pouvaient être porteurs de l’antigène Australie.

L’ICR a cédé à la firme pharmaceutique Merck and Co les droits d’exploitation du brevet concernant le vaccin contre l’hépatite B. Hilleman et ses collaborateurs de chez Merck ont produit un vaccin purifié, fabriqué à partir d’un mélange de plasmas de porteurs de virus de l’hépatite B (11). Ce mélange contenait les 2 sous-types adr et ayw, dans les proportions respectives de 89 et 11 p. 100. Les particules d’HBs Ag étaient purifiées par centrifugation isopycnique en chlorure de césium (2 fois) et par ultracentrifugation en gradient de sucrose (1 fois). La préparation finale, utilisée dans leurs essais, était ajustée pour contenir 20 μg/ml de protéines (déterminés par la méthode de Lowry) et traitée pendant 72 heures à 36 °C avec du formol au 1/4 000. Les préparations ont été testées sur des chimpanzés de 10 à 20 kg. 6 de ces animaux reçurent 1 ml de vaccin en intraveineux. Aucun animal de ce groupe ne développa d’hépatite.

6 chimpanzés contrôles, qui reçurent 1 000 doses infectantes du virus B vivant, atténuèrent l’hépatite. Le groupe de Merck a aussi testé le pouvoir immunogène du vaccin sur des cobayes. Ils montrèrent que 2 μg administrés une fois en sous-cutanée provoquent l’apparition d’anticorps. Quand 3 doses successives étaient administrées, la dose nécessaire par injection était seulement de 0,5 μg. On obtenait une séroconversion chez plus de 50 p. 100 des animaux, après 3 doses de 2 μg. 4 des 6 animaux qui reçurent chacun 3 injections de 20 μg répondirent. Le vaccin apparaît sûr puisque aucun des chimpanzés vaccinés ne présente d’augmentation des SGPT, d’apparition d’HBs Ag ou d’anticorps dirigés contre la partie centrale de l’antigène (anti-HBc), tous signes considérés comme des témoins de l’infection.

Pendant que Merck essayait son vaccin, Purcell et Gerin, à l’Institut national de la Santé, dans le Maryland, testaient de leur côté un vaccin purifié fabriqué d’une manière analogue. Des vaccins ayw et adr ont été purifiés chacun à partir d’une unité de sang de porteur asymptomatique. Les deux vaccins de Purcell et Gerin et celui de Merck sont constitués principalement de particules de 22 nm purifiées par centrifugation. Le vaccin de Purcell et Gerin est inactivé par incubation dans du formol au 1/2000, 96 heures à 37 °C. La concentration des protéines dans les vaccins est ajustée à 20 μg/ml (Lowry). Le vaccin de Purcell et Gerin diffère de celui de Merck essentiellement par le fait qu’il est stabilisé avec de l’albumine sérique humaine à 1,4 p. 100.

Purcell et Gerin utilisent une dose immunitaire de 0,1 ml par voie sous-cutanée, suivie par une injection de rappel avec la même dose en sous-cutanée un mois plus tard (12). 7 chimpan-
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Les chimpanzés, dont 3 contrôles, sont utilisés pour ces tests. Un des chimpanzés contrôlés, reçoit en intraveineux 1 ml de sérum HBv (sous-type ayw) qui a servi à la fabrication du vaccin, un second reçoit la sérum-albumine humaine utilisée comme diluant et le troisième ne reçoit pas d’injection. 3 chimpanzés reçoivent le vaccin purifié du sous-type ayw, 2 reçoivent le vaccin après inactivation par le formol et le troisième avant inactivation. Le quatrième animal est vacciné avec le vaccin adr inactifié par le formol. Les séums des 7 chimpanzés sont testés pour les SGPT, l’HBs Ag, l’anti-HBs et l’anti-HBc. Seul le chimpanzé inoculé avec le sérum HBs Ag + ayw devient infecté avec augmentation des séums, apparition d’anti-HBc et d’HBs Ag. Aucun des 6 autres animaux ne présente ces modifications. Les anticorps dirigés contre HBs Ag (anti-HBs) sont détectés (par radio-immunologie) chez les 4 animaux vaccinés. Des titres du 1/32 au 1/64 sont trouvés par hémagglutination passive. Les animaux vaccinés et celui qui n’a reçu aucune injection, sont inoculés avec HBv (souche MS-2, sous-type ayw), à une dose calculée pour entraîner l’hépatite chez la moitié des chimpanzés (103,5 doses infectantes (DI50), 24 semaines après la dernière injection du vaccin. Les échantillons de sang sont recueillis 2 fois par semaine, après cette infestation. Chez les 2 animaux contrôlés (celui qui n’a reçu aucune injection et celui qui a reçu le diluant) apparaissent HBs Ag et anti-HBc. Au contraire, aucun des animaux immunisés n’a d’HBs Ag dans son sérum ou ne développe de signes d’hépatite. Celui qui a reçu le vaccin hétérologue adr, répond mal et a initialement des signes sérologiques d’infection (un titre faible d’anti-HBs suivi par l’apparition d’anti-HBc), mais sans signes biochimiques d’atteinte hépatique. Cette réponse rappelle l’infection cliniquement latente de l’homme sans signes biologiques, mais avec une réplication virale.

En juin 1976, des expérimentateurs de chez Merck rapportent leurs travaux de protection chez le chimpanzé (13). Ils trouvent que 3 doses de vaccin (voir ci-dessus) protègent les chimpanzés contre 1 000 doses infectantes de virus B vivant donné en intraveineux. 6 chimpanzés sont vaccinés avant l’inoculation et 5 autres non vaccinés servent de contrôle. Les animaux sont examinés pendant 24 semaines. Aucun des animaux vaccinés ne présente de signes cliniques d’hépatite.

Les anticorps dirigés contre le sous-type a, paraissent suffisants pour la protection. Murphy et coll. (14) ont indiqué que, dans les expériences où les chimpanzés étaient inoculés avec des préparations non purifiées d’HBs Ag contenant différents sous-types, les animaux devenaient infectés et développaient l’HBs Ag et éventuellement l’anti-HBs, correspondant à l’inoculation initiale. Des études complémentaires faites par Barker et coll. (15) ont montré que les chimpanzés convalescents sont protégés de l’infection par des préparations de virus de l’hépatite B ayant un sous-type différent. Bien que ces résultats paraissent indiscutables, il est peu souhaitable que des vaccins soient préparés à partir d’un seul sous-type. Ou bien des mélange de différents sous-types seront préparés, ou bien les vaccins seront fabriqués à partir des sous-types les plus souvent rencontrés dans le pays où le vaccin sera utilisé.

Maupas et ses collègues à Tours, en France, ont aussi rapporté leur expérience de vaccination. Ils purifient le vaccin par chromatographie d’affinité, en utilisant un anti-HBs couplé par du bromure de cyanogène au sepharose 4B (16). Le sérum contenant l’HBs Ag de sous-type ayw avec un titre en électrosmérèse supérieur ou égal au 1/16 est passé sur la colonne. L’HBs Ag est élué avec NaSCN à la concentration de 3 ml/litre à pH 7,4. La première fraction d’HBs Ag éluée est passée à travers une seconde colonne contenant des anticorps anti-sérum humain couplés au sepharose. Le second passage est nécessaire pour éliminer les traces de protéines éluées avec l’HBs Ag à partir de la première colonne. Le matériel purifié, standardisé à une concentration en protéines de 70 μg/ml a un titre en électrosmérèse de 1/2, en hémagglutination passive au 1/512 et en radio-immunologie au 1/1000. Des traces d’IgG sont encore détectables. Le vaccin est ensuite inactifié dans du formol à 0,1 p. 100 pendant 48 heures, à 37 °C et une semaine à +4 °C. Administré à 5 chimpanzés, il est efficace, c’est-à-dire qu’aucun des animaux n’a de signe d’infection par le virus de l’hépatite B. Maupas a ensuite vacciné 30 malades et 73 membres du personnel soignant de l’unité d’hémodialyse de Tours. Chaque individu vacciné reçoit 2 injections de 1 ml de vaccin en sous-cutanée dans le mois. Il rapporte que 95 p. 100 du personnel soignant et 60 p. 100 des malades développent des anticorps anti-HBs. Les membres du personnel soignant et les malades sont suivis pendant 1 an. Aucun membre du groupe vacciné ne présente d’hépatite clinique ou ne devient HBs Ag+. 50 p. 100 des membres du personnel soignant non vaccinés et 60 p. 100 des malades deviennent HBs Ag+. Dans le groupe des gens qui ont été apparemment protégés, se trouvent 11 individus qui n’ont pas développé d’anticorps anti-HBs après la vaccination. L’interprétation donnée par Maupas est que le protection pourrait survenir même en l’absence d’anticorps anti-HBs détectables. Il postule que l’immunité cellulaire peut jouer un rôle protecteur. Maupas est le premier à utiliser l’hydroxyde d’alumine.
comme adjuvant au vaccin. L'utilisation de l'adjuvant entraîne une séroconversion plus rapide.

Brzosko et coll., dans *Hepatitis Scientific Memoranda*, en mars 1976, rapportent des résultats préliminaires avec un vaccin purifié de façon semblable. Leur premier lot de vaccins est purifié par chromatographie à partir de sérums adr et ayw, standardisés pour contenir 200 μg/ml de protéines et inactivés avec du formol au 1/100 pendant 168 heures (la température d'activation n'est pas précisée). Le formol est enlevé par dialyse. 3 chimpanzés sont inoculés avec des doses massives de vaccin pour tester son innocuité. Chaque animal reçoit 10 ml de vaccin contenant 2 mg d'HBsAg par voie intramusculaire et intraveineuse. 9 semaines plus tard, les animaux reçoivent des injections de rappel de 5 ml de vaccin en intramusculaire. 2 mois après le 1er rappel, les animaux sont injectés avec 0,1 ml de vaccin en intramusculaire. 4 semaines après la dernière injection, ils sont inoculés avec HBV fait d'un mélange de sous-types (103.25 doses infectantes (DI)50 adr et 103.25 (DI)50 ayw). 10 semaines plus tard, ces mêmes animaux sont utilisés pour tester l'immunogénicité d'un second lot de vaccin. Ce lot est préparé à partir d'un sérum contenant HBsAg précipité par le sulfate d'ammonium, suivi d'une digestion par la pepsine et d'une concentration sur un système Amicon DC 30 équipé avec une membrane XM 100. Les faits rapportés dans ce résumé sont difficiles à interpréter. Les auteurs ont déclaré que l'HBsAg a été détecté chez chaque animal une fois seulement, dans l'échantillon prélevé une semaine après une nouvelle injection de vaccin faite 10 semaines plus tard. Anti-HBc n'est jamais trouvé chez aucun des animaux et l'anti-HBs est détecté chez tous les animaux après la première semaine. Ils concluent que le vaccin, fabriqué selon ce procédé, est sans danger.

Nous avons depuis longtemps suggéré que l'antigène Australia pourrait contenir des spécificités antigéniques analogues ou identiques à certaines protéines du sérum humain et à d'autres constituants antigéniques de l'hôte et que de telles spécificités antigéniques sont polymorphes au sein de l'espèce humaine. Des études ont donné des résultats contradictoires à ce sujet. Le vaccin est purifié à partir du sérum humain et des contaminants possibles pourraient inclure des protéines sériques. Ainsi se trouve soulevée la possibilité que la vaccination entraîne le développement d'anticorps dirigés contre des protéines de l'hôte, ce qui pourrait avoir un effet indésirable. Un problème analogue pourrait exister chez les patients qui reçoivent des transfusions de sang ou d'autres vaccins qui sont produits sur cultures de cellules humaines. A notre connaissance, de tels effets n'ont pas été observés mais il est aussi peu probable que le problème ait été étudié de façon systématique chez les patients transfusés et chez ceux qui ont été vaccinés à partir de vaccins humains. En raison de ce problème théorique, à savoir que le vaccin pourrait contenir des acides nucléiques de l'hôte et/ou des antigènes humains d'origine cellulaire ou sérique, des vaccins polypeptidiques ont été proposés (17, 18). Dans ceux-ci, l'HBsAg isolé serait fractionné par des traitements enzymatiques ou autres, dans l'espoir que les antigènes induisant la production d'anti-HBs et d'anticorps dirigés contre l'hôte se trouvent dans des fractions différentes. Il est cependant possible aussi que ces 2 éléments soient dans la même fraction et des tests appropriés devraient être mis en œuvre pour préciser s'il en est ainsi. Dans cette optique, Melnick et coll. (16) ont montré que la migration de cellules de l'exsudat périnéral de cobaye immunisé avec une fraction polypeptidique de 24 000 dalton était inhibée par le sérum humain normal. Ceci implique que les constituants de l'hôte ou des déterminants du sérum normal font partie de la macromolécule de l'HBsAg. Une telle possibilité a été postulée la première fois par Millman et coll. (19), puis soutenue plus tard par Neurath (20). Un autre point, concernant l'utilisation de vaccins préparés ainsi est qu'ils pourraient avoir une plus faible antigénicité que les vaccins préparés à partir de l'antigène de surface intact et que de plus grandes quantités d'un vaccin, dont la production est onéreuse, pourraient être nécessaires.

En plus des études rapportées ici, des recherches sur le vaccin sont en cours au Japon, dans la République populaire de Chine, en Suède, aux Pays-Bas et probablement ailleurs.

Lustbader et coll. (21) ont conçu une étude destinée à tester un vaccin contre l'hépatite B dans une population à haut risque (unité d'hémodialyse rénale) qui peut servir à tester la valeur du vaccin. Ceci a l'avantage d'utiliser une population test relativement petite et de ne pas nécessiter des témoins en parallèle.

Les résultats des études publiées à ce jour et résumées ici, sont encourageants. Pour que les vaccins soient acceptables, ils doivent être efficaces et sûrs. Leur efficacité et leur innocuité doivent être tout à fait convaincantes pour satisfaire aux règles utilisées par les organismes officiels, tels que la « Food and Drug Administration » aux USA, pour autoriser la mise sur le marché.

Si le vaccin est accepté, il peut trouver alors de nombreuses applications. L'hépatite B est un...
problème important dans les régions du monde développées médicalement, mais aussi et encore davantage dans l'Afrique sub-Saharienne, dans certaines régions d'Asie et d'Océanie et ailleurs où les taux d'infestation peuvent être extraordinairement élevés. Dans ces pays, les maladies chroniques du foie et le cancer primitif du foie peuvent être très fréquents et il y a des raisons de penser qu'ils peuvent être associés à une infection persistante par HBV. Si ces hypothèses sont confirmées, les programmes de vaccination contre l'hépatite B pourraient connaître d'importantes conséquences, notamment la prévention d'un cancer fréquent et mortel et ceci pourrait constituer un exemple d'un vaccin anticancéreux.*

* Une revue sur l'utilisation de gammaglobulines avec un titre élevé d'anticorps anti-HBs pour la prophylaxie passive de l'hépatite B, récemment publiée, pourrait intéresser les lecteurs de cet article (22).

(Perspectives de la vaccination contre le virus de l'hépatite B — 1951)

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e Antigen in Hepatitis B Virus Infected Dialysis Patients: Assessment of Its Prognostic Value

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Many investigators consider the presence of e antigen (HBeAg) valuable in predicting which patients with acute hepatitis B are at risk of remaining infected and developing chronic liver disease. We tested this hypothesis in a retrospective study of serial samples from patients undergoing long-term hemodialysis. We found HBeAg in the early phase of all hepatitis B virus (HBV) infections. There was no significant difference between transiently and persistently hepatitis B surface antigen (HBsAg)-positive persons with regard to the frequency of HBeAg during the first 3 months of HBs antigenemia. Thus during the early period of viral activity, the presence of HBeAg is of no prognostic value in determining chronicity of HBV infection. We believe the disagreement on the prognostic value of HBeAg is a reflection of variations in time and frequency of sampling. The HBeAg remains, however, a useful indicator of potential infectivity of HBsAg(+) persons.

There are two major classes of responders to infection with hepatitis B virus (HBV): those persons who make antibody to hepatitis B surface antigen (anti-HBs), clear the virus, and are thereby only transiently hepatitis B surface antigen (HBsAg) positive; and those who are unable to produce anti-HBs and are persistently positive for HBsAg. The e antigen system was first recognized in sera containing HBsAg by Magnus and Espmark (1). The e antigen (HBeAg) is immunologically distinct from hepatitis B surface and core antigens and is a marker of increased infectivity of HBsAg-positive sera (2-5). In addition, Nielsen, Dietrichson, and Juhl (6) have suggested that HBeAg is of prognostic value in HBsAg-positive acute viral hepatitis in predicting progression to chronic hepatitis or cirrhosis. A number of reports have supported their hypothesis (7-9), but some investigators have presented data to the contrary or at least urged caution because the correlations are not absolute (10-14).

Testing samples collected monthly from patients with end-stage renal disease undergoing chronic hemodialysis, we retrospectively assessed the value of HBeAg in predicting which patients will become transiently or persistently HBsAg(+) persons. We also determined the frequency of occurrence of HBeAg in these groups and defined its time of appearance and duration of positivity relative to other indicators of HBV infection.

Materials and Methods

Assays

Sera were tested for HBsAg by counterelectrophoresis, immunodiffusion, and radioimmunoassay (Austria II, Abbott Laboratories, North Chicago, Illinois). Antibody to HBsAg(anti-HBs) was assayed by passive hemagglutination (15); titers were determined from twofold serial dilutions incubated with HBsAg coated human erythrocytes (Electronucleonics, Bethesda, Maryland). Antibody to hepatitis B core antigen (anti-HBc) was measured using counterelectrophoresis and immunodiffusion methods (16). Both HBeAg and anti-HBe were detected by rheophoresis in 0.8% agarose plates (Abbott Laboratories) (17). Serum glutamic pyruvic transaminase (SGPT, or serum alanine transferase) activity was measured by Henry's modification (18) of the method of Karmen and colleagues (19) and recorded in Karmen units (KU).

Patient Groups

The general patient population, the epidemiology of hepatitis B infection, and the dialysis regimen at a community-based dialysis unit, Delaware Valley Artificial Kidney Clinic (DVAKC), have been described in detail in previous reports (20-22). Sera were obtained monthly for SGPT measurements and HBsAg and anti-HBs testing. Sequential serum samples from nine transiently and nine persistently HBsAg-positive patients were obtained for periods of 14 to 42 months. All persons in these two groups began dialysis therapy HBsAg(-) and became HBsAg(+) while at the DVAKC. Serum samples from another group of six chronic carriers who were HBsAg(+) at admission to the DVAKC were collected for 16 to 63 months. After the initial HBeAg findings in the transiently HBsAg(+) patients, a second group of 10 transiently HBsAg(+) patients was tested for HBeAg. Finally, a cross-sectional sample of 32 HBsAg(+) patients was analyzed to find the point-prevalence of HBeAg in this group. This group consisted of all persons HBsAg-positive by radioimmunoassay at one randomly selected monthly testing of all DVAKC patients.

Statistical Tests

We used 2 x 2 chi-square tests to compare the frequencies of HBeAg in the various groups. Mann-Whitney tests were used to compare the HBeAg-positive and -negative patients in the point-prevalence sample with respect to age, transaminase level, and duration of HBsAg positivity (23).

Results

Table 1 summarizes the HBeAg results for the serial samples from transient and chronic carriers of HBsAg. All 28 patients in our study who converted to HBsAg(+) at this dialysis unit were also HBeAg(+) for at least one
monthly serum testing, regardless of their eventual response to HBV. Only two patients were never HBeAg(+) during the analysis period. They were HBsAg(+) on arrival at DVAKC, and it is not possible to know if they were previously HBeAg(+). The results of testing sera from the second group of patients who were transiently HBsAg(+) confirmed those for Group 1. The HBeAg was found in the serum of every person, and when Groups 1 and 2 were compared the percentages of HBeAg(+) specimens during HBs antigenemia were not significantly different.

A comparison of the HBsAg(+) samples from the persistent carriers who converted to HBsAg(+) at the DVAKC with the HBsAg(+) samples from both transiently positive groups showed a significantly higher frequency of HBeAg in the long-term carriers (P < 0.01). The two groups of persistently positive patients were also different from each other with regard to the frequency of HBeAg-positive samples (P < 0.01). The percentage of HBeAg(+) samples was lower in the group of patients who were HBsAg(+) before dialysis at the DVAKC.

By examining all HBsAg(+) persons at one randomly selected monthly testing of DVAKC patients, the point-prevalence of HBeAg among persistently HBsAg(+) patients was found to be 63% (20 of 32). Additional testing indicated that 29 of these 32 persons (91%) were HBsAg(+) at some time during treatment at the DVAKC. All three who lacked HBeAg came to the unit HBsAg(-). (Two of these three patients are included in the sequential study.) The HBeAg(+) samples had slightly higher HBsAg titers by counterelectrophoresis (median = 1:256) compared to HBsAg(-) samples (median = 1:64). The HBeAg(+) patients also had significantly higher SGPT values (Mann-Whitney U test, P < 0.01), although only one person had an elevation greater than 40 KU. The HBeAg-positive and -negative persons did not differ significantly with regard to sex (Fisher's exact 2 x 2 test) or age (Mann-Whitney U test). Additional testing for HBsAg showed that all persons in the point-prevalence sample were long-term carriers, but, as illustrated in Figure 1, the HBeAg(-) patients had been HBsAg(+) significantly longer than those who were still HBeAg(+).

Figure 2 shows the presence of HBeAg in serial samples from the persistently HBsAg(+) patients in Table 1. Among the nine persons for whom the time of conversion to HBsAg(+) is known, seven were HBsAg(+) for at least 2 years, and of these persons five remained HBeAg(+). Of the six HBsAg carriers who were HBsAg(+) on arrival at the dialysis unit, three were HBeAg(+) for at least 2 years while the other three were either only briefly positive or never had HBeAg or anti-HBe. One patient was followed for more than 5 years and never had detectable HBeAg. Thus individuals may remain chronic HBsAg carriers long after the loss of detectable HBeAg. In addition, some persons who were HBeAg(+) for long periods were intermittently HBeAg(-).

The time of appearance of HBeAg relative to the first HBsAg-positive sample (by radioimmunoassay) can be seen graphically in Figure 2, top, and in Figure 3. Samples obtained 1 and 2 months before HBsAg positivity were tested for HBeAg and were uniformly negative. In the chronic carriers of HBsAg, HBeAg positivity followed the detection of HBsAg by radioimmunoassay by 1 month in five cases and appeared concomitantly in four others. Using the less sensitive counterelectrophoresis and immunodiffusion methods to detect HBsAg, no difference in time of appearance of HBsAg and HBeAg was observed. The HBeAg was present slightly before or at the same time as anti-HBc and the first transaminase elevation. All but one patient had elevated SGPT levels; three had consistently high levels for 1 year or longer. In two of these three patients, the SGPT levels dropped to normal values at the same time that HBeAg was no longer detected.

Six of nine transiently HBsAg(+) persons (Figure 3, left) were positive by radioimmunoassay 1 month before HBeAg positivity; in the other three, both antigens appeared concomitantly. The HBeAg was also first detected at the same time as or shortly after the first HBsAg(+) sample (detected by counterelectrophoresis in this group). The HBeAg occurred about the same time as anti-HBc and was usually positive before the peak SGPT, although in two cases the peak coincided with the appearance of HBeAg. A month after the peak SGPT only two of nine patients were still HBeAg(+). All of these patients showed only transient elevations in SGPT. As previously reported (22), the transiently HBsAg(+) patients generally had higher transaminase elevations than

| Table 1. HBeAg Testing of Sequential HBsAg(+) Samples from Dialysis Patients |
|------------------|----------|--------|--------|--------|
|                  | Patients | Samples | HBeAg(+) | Patients |
|                  |          | Tested  |         |        |
| Transiently HBsAg(+) |
| Initial group    | 9        | 42      | 21 (50.0) | 9 (100) |
| Second group     | 10       | 45      | 18 (40.0) | 10 (100) |
| Persistently HBsAg(+) |
| Converted at clinic arrived positive | 9 | 226 | 183 (81.0) | 9 (100) |
|                   | 6        | 189     | 74 (39.2) | 4 (66.7) |

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Those chronically HBsAg positive. The second group of transiently positive patients had similar patterns, with the appearance of HBeAg after that of HBsAg by 1 or 2 months. As in the other groups, HBeAg was not detected when HBsAg was positive only by radioimmunoassay.

No transiently HBsAg(+) patient was HBeAg positive longer than 4 months, whereas most long-term carriers of HBsAg did not begin to lose detectable levels of HBeAg until after 18 months. Thus HBeAg clearly persists for a longer time in chronic carriers of HBsAg. During the first 3 months of HBs antigenemia, however, there was no significant difference in the frequency of HBeAg between chronic carriers and those transiently HBsAg(+).

Ten of the 19 transiently HBsAg(+) patients developed anti-HBe during the year after the disappearance of HBsAg and HBeAg. None of the chronic carriers of HBsAg had detectable anti-HBe after the apparent loss of HBeAg.

Discussion

Hepatitis B virus infections are common among persons undergoing long-term hemodialysis (24-27), and many of these patients are HBeAg(+) as well as HBsAg(+) (1). In an earlier study we showed that the probability of a patient who became HBsAg(+) in this clinic becoming a chronic carrier was 62.8% and rose to 88.5% if the patient was antigenemic for 5 consecutive months (22). If infected with HBV, men were more likely to become chronic carriers and women more likely to develop anti-HBs (22). Age, race, or type of underlying kidney disease could not be used to predict which patients were at greatest risk of becoming chronic carriers of HBsAg.

In the past few years, evidence has been accumulating that the presence of HBeAg may predict which patients with acute hepatitis are more likely to develop chronic liver disease, although this has recently been questioned. We examined the sera of patients converting to HBsAg

while on dialysis to determine if the presence of HBeAg indicated which patients would become chronic carriers and would therefore be more likely to develop chronic liver disease. A total of 28 patients were studied during the first weeks of HBs antigenemia, and all 28 were HBeAg(+) for at least one monthly sample. This group included 19 transiently HBsAg-positive patients. Thus the presence of HBeAg does not indicate which persons will become chronically HBsAg(+).

The temporal relation of the testing for HBeAg to the onset of detectable HBsAg must be carefully defined. Lofgren, Myhre, and Nordenfelt (8) have examined serial samples from patients who were either transient or chronic carriers and found only three of 21 patients with transient HBs antigenemia to be HBeAg(+) as compared with 16 of 18 HBsAg(+) carriers. The most probable reason for the difference from our results is that the first samples they tested were already HBsAg positive, whereas in most of our cases the sample 1 month before conversion was available for testing. We could therefore ascertain the first appearance of HBsAg more precisely. We showed that the transiently HBsAg-positive patients had HBeAg only briefly; thus Lofgren's patients may already have lost the antigen before testing. Other reports also indicate the importance of time of sampling. Gibson and Ruparelia (28) found that 15 of 16 patients tested within the first week of jaundice were HBeAg(-), whereas none of 21 patients with acute hepatitis B who were tested later than 1 week after the onset of jaundice was HBeAg(-). Lam, Redeker, and Mosley (29) showed that specimens obtained early in the illness were more frequently HBeAg(-) than later samples; and Aikawa and colleagues (30) recently found that all seven patients studied were HBeAg(-) during the early stage of infection.

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second possibility for the difference in findings is that our patients were undergoing long-term dialysis and therefore were not in good health before the HBV infection. Cappel, DeCuyper, and Van Beers (14) recently found a higher frequency of HBeAg positivity in hemodialysis patients with acute hepatitis than in nondialyzed patients. Because all their patients had clinical and biochemical signs of acute hepatitis when they entered the study, it is not possible to make a direct comparison with our data or learn about the duration of HBeAg positivity in their patient groups.

As clearly pointed out by Nielsen, Dietrichson, and Juul (31), there is difficulty in excluding chronic liver involvement without a biopsy because biochemical test findings may be misleading. We recognize the problem in assuming that the transiently HBsAg (+) patients did not develop chronic liver disease; however, the rapid fall of SGPT to normal levels and the fact that all but one patient became anti-HBs (+) suggest that this is a fair assumption.

We have also shown that chronic carriers of HBsAg may lose detectable HBeAg; in this group the loss of HBeAg does not seem to predict the loss of HBsAg. However, more sensitive detection methods may show that HBeAg is still present in these persons. The relative insensitivity of the rheophoresis method may also account for the intermittent periods in which HBeAg is present without detectable HBeAg (Figure 2). Our results are in accord with those of Vogten and co-workers (9), who showed that the disappearance of HBeAg during the course of chronic active liver disease did not always lead to a better prognosis, clearance of HBsAg, or production of anti-HBs. In general, however, we have found that those carriers who eventually lose HBeAg have lower SGPT values, a finding consistent with those of others who have found evidence of more liver damage in HBeAg (+) persons (12, 32-34).

At present the nature and origin of HBeAg are unclear. Magnius and associates (35) have suggested that HBeAg may be produced as a host response to active HBV infection, possibly as an idiotypic determinant on IgG (36). Another possibility is that HBeAg may be needed for complete viral particles as suggested by the recent data of Lam, Tong, and Rakela (37), who released HBeAg from detergent-treated Dane particles. The time of appearance of HBeAg relative to other indicators of HBV infection does not allow us to evaluate these possibilities. More sensitive detection methods for HBeAg may help clarify this issue.

The regular testing of hemodialysis patients at the DVA KC usually allowed us to find evidence of HBV infection before clinical symptoms became apparent and before serum transaminase concentrations became elevated. Thus we were able to show that all HBV-infected persons are at least transiently HBeAg positive. The difference in opinion about the prognostic value of HBeAg is likely to be related to the variations in frequency and time of sampling. The time of sampling relative to the onset of disease is critical when only a single specimen is tested.

Sherlock (38) has pointed out that the persistence of HBeAg in patients recovering from type B hepatitis indicates a poor prognosis, and we have shown that HBeAg is present longer in chronic carriers. Thus only during convalescence does HBeAg have any value in predicting chronic HBV infection, and by this time in the disease course it is apparent that HBsAg is also persisting. The importance of HBeAg testing therefore lies in its role as an indicator of potential infectivity of HBsAg (+) patients and their sera. This has been documented both by transmission data (2-5) and by studies from our laboratory and others showing the close association of HBeAg to the presence of the intact virus and viral replication (17, 39, 40).

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VIRUSES SIMILAR TO HEPATITIS B VIRUS (ICRONS)

Baruch S. Blumberg, M.D.*

In 1971, on the basis of the unusual clinical and epidemiologic characteristics of hepatitis B virus, we postulated the existence of a class of viruses that we termed icrons. An increased understanding of the molecular biology of hepatitis B virus resulted in the discovery, by Summers and his colleagues, of the woodchuck hepatitis virus. This virus is common in the North American woodchuck (Marmota monax) and is associated with primary cancer of the liver in this animal. Subsequently similar viruses were found in Beechey ground squirrels in California by Marion and her coworkers and domesticated (Pekin) ducks from the United States by Mason and his coworkers. In the latter the virus infects the liver and presumably is associated with disease of this organ.

The discovery of additional viruses similar to hepatitis B virus in animals other than man and their association with cancer of the liver encourages the continuing search for other virus-cancer associations for which prevention methods might be effective. Hum Pathol 12:1107-1113, 1981.

The association of "Australia antigen" with hepatitis virus was found in 1967. By 1970 it was recognized on the basis of epidemiologic, serologic, and clinical evidence that it was an unusual infectious agent. This was in large part a consequence of the history of its discovery. For several years we had been investigating serum protein polymorphisms in humans and lower animals. The term polymorphism, as used in population genetics, refers to two or more inherited discontinuous forms of a trait in a population of a species in which the form of the trait present in lowest frequency would not be maintained in the population on the basis of simple recurrent mutation alone. The term polymorphism, as originally introduced by the British lepidopterist E. B. Ford, in addition to describing the numerical relationships of the distinct inherited types, carried with it the explanation that the several forms of the trait were retained in the population as a consequence of differential survival values. For example, if the heterozygote were at a selective advantage, both homozygous forms would be retained, even if one homozygote were at a disadvantage. The most frequently cited example in humans is the sickle cell trait. Individuals who are heterozygotes for the Hb^s gene (and have both sickle and normal adult hemoglobin) have relative protection against infection with falciparum malaria and maintain both genes in the population despite the disadvantage to the homozygote Hb^s individuals who usually develop life shortening sickle cell anemia.

Independent of the explanation for their existence, many polymorphisms are known in humans and other animals. They include the well known red blood cell antigens (e.g., ABO and Rh), many serum proteins (haptoglobin, transferrins, Ag lipoproteins), red blood cell enzymes, and antigens on the white blood cells (the HLA system). Often the inherited differences are antigenic, and this has important clinical consequences. Patients transfused with whole blood must be given red blood cells whose ABO and Rh antigens match their own; otherwise a serious immune transfusion reaction will develop. When organs are transplanted from one individual to another, the donor and recipient are matched for as many HLA antigens as possible, although the contribution of this antigenic locus to transplant rejection may not be as great as it was originally thought to be.

After identification of Australia antigen as the surface coat of an infecting agent, it still was useful to consider the virus and the human immunologic response to infection with it as a polymorphic system, and this led to curious insights into the action of hepatitis B virus. There are several features of Australia antigen (hepatitis B surface antigen, HBsAg) and hepatitis B virus that are similar to those of a serum protein polymorphism. The distribution of asymptomatic individuals who are persistently infected with hepatitis B virus (chronic carriers) and those who develop antibody against the surface antigen (anti-HBs) mimics the distribution of a serum protein polymorphism. After infection, an individual appears to have the capacity to either become a persistent carrier or develop anti-HBs. It is very rare for an individual to have both these responses simultaneously. There is evidence that the propensity for becoming a carrier is under some form of genetic control, but it does not appear to follow a simple pattern of segregation.

The geographical distribution of subtypes of HBsAg may also be explainable by a genetic mecha-
nism. Certain subtypes are restricted to particular geographic regions. For example, subtypes adr are found commonly in Southeast Asia and Oceania but rarely in the United States and Europe. These specificities do not appear to spread rapidly to other geographic regions. One may conjecture that although infection may occur, only residents of the region with an inherited ability to become persistent carriers of their particular local specificity will become carriers. This may mirror the remote peopling of a country. For example, the HBsAg carriers in the Ryukyu Islands (to the south of the Japanese main islands) and in the southern portions of the main islands of Japan have a distribution of specificities similar to those in Indonesia and Malaysia whence they were settled; central and northern Japan reflects the peopling of these regions from central Asia via Korea and northern Japan. This could be explained by the existence of genes that rendered an individual susceptible to persistent infection with a particular specificity and the preservation of these genes in the gene pools of populations.

Independent of the validity of the genetic hypothesis, within an area where hepatitis B virus is endemic there will be a large number of people (5 to 20 per cent) who are carriers of the virus, and these individuals have large numbers of particles containing HBsAg in the peripheral blood. An even larger number (40 to 70 per cent) have antibody against the surface antigen (anti-HBs). The virus can be identified in the sera of apparently normal people by the use of immunologic techniques in which one human serum is reacted against another. The potential reagents are very common, and this simplifies the identification of viruses and the acquisition of reagents.

There are striking differences in the way in which humans react to infection with the virus. As already discussed, most of those infected either develop anti-HBs or become carriers, and neither of these groups becomes actually ill. Acute hepatitis develops in a small percentage of the population. Most of these people recover completely, but some (5 to 10 per cent) develop chronic hepatitis, which can progress to life shortening, posthepatitis cirrhosis, and some chronically infected individuals develop primary hepatocellular carcinoma. (This process is described in detail in the accompanying article by W. T. London.)

There are interesting concepts that emerge from consideration of the interaction of hepatitis B virus and its host as an antigenic polymorphic system. For example, if the infectious agent is construed to be a polymorphism, one can conjecture that the “host” protein on the agent is not necessarily that of the host from which it is isolated, but could be related to the previous host or hosts; i.e., it could be an isoantigen. If this concept is correct, we would expect to see interactions between the infectious agent and the host that are analogous to transfusion reactions. In blood transfusion, if the patient has group A blood and receives type A blood, no antibodies will develop. If the patient receives type B, antibodies will form and a transfusion reaction can occur. Similarly, if a host is infected by an agent that has the same serum protein specificity as the host, no antibody will form and presumably invasion by the organism can occur. If the specificities are different, antibodies will form, and this may or may not result in invasion and symptoms of one or more different kinds of disease. This could include antigen-antibody complexes.

On the basis of what appeared to be differences between hepatitis B virus and other viruses, for heuristic reasons we suggested a group name, Icron, for hepatitis B virus and other agents like it that might be found in the future. The name consists of an acronym for the Institute for Cancer Research, where the virus was discovered, with a neuter Greek ending. It also carries the implication of a particle. The motivation for assigning the name was to focus attention on the discovery of new viruses by emphasizing the unusual characteristics of hepatitis B virus.

**MOLECULAR BIOLOGY OF HEPATITIS B VIRUS**

The clinical and epidemiologic characteristics of hepatitis B virus suggested that it might be representative of a distinctive group of infectious agents. Following the identification of the whole hepatitis B virus virion and the characterization of the molecular biology of the virus, it became even more apparent that it was different from other viruses. The molecular biology of the hepatitis B virus is reviewed by Dr. London in this symposium but is summarized briefly here to indicate the characteristics to be looked for in viruses of the Icron group.

1. There are three particle forms of hepatitis B virus. The whole virion (initially called the Dane particle after the person who first described it) is 42 nm. in diameter (range, 40 to 50 nm.). There is a central core particle about 24 nm. in diameter that contains DNA and a specific DNA polymerase. The surface antigen (HBsAg) is antigenically distinct from the core antigen (HBcAg). A second smaller (22 nm.) particle is composed entirely of surface antigen. A third particle is of the same diameter as the small particle but is elongated and has a variable length up to about 500 nm. The surface antigen particles (both small and elongated) may occur in large numbers. It has been estimated that in some carriers, up to 1 per cent of the serum protein is surface antigen.

2. The hepatitis B virus DNA has unusual characteristics. It is circular and double stranded and has about 3200 base pairs. This is the smallest number of base pairs in any known DNA virus. Neither of the strands is closed. One of the strands is of variable length and composes about 50 per cent or more of the more complete strand. That is, the DNA is single stranded in part and double stranded in part, and there is considerable individual variation in the length of the single stranded portion. At the time of discovery this was a unique characteristic of the virus.

3. DNA polymerase present in the core acts in vitro to complete the single stranded region. The virus does not require any polymerase from the host for this purpose; this is also a unique characteristic.
4. The virus cannot be grown in conventional tissue cultures. There are, however, some interesting tissue culture systems that have been developed. Alexander obtained tissue from a South African patient with cancer of the liver and was able to induce this tissue to grow by using tissue culture techniques. It has been subjected to passage many times, and additional cultures of this kind have been prepared. The tissue culture fluid contains HBsAg, and viral DNA is integrated into the genome of the cells. However, whole virus particles are not produced. Growth of a virus in tissue culture is usually considered to be essential to virus research. Although this has not been done for hepatitis B virus, there is a great deal known about the virus. This includes preparation of a vaccine, the development of highly sensitive methods for its detection, the analysis of the nucleic acid sequence of the genome (one of the few human viruses that have been so characterized), and the cloning of the sections of the gene that produce core and surface antigen (see the London article for references).

DISCOVERY OF WOODCHUCK HEPATITIS VIRUS

Early in our studies of the hepatitis B virus, in order to carry out inoculation studies we required an animal other than man that could be infected with the virus. We surveyed sera of about 30 different species to identify animals that might have become infected “naturally.” HBsAg was found only in nonhuman primates but not in any of the usual laboratory animals. We were given several primates that had been infected with hepatitis B virus, and these were housed at the Penrose Research Laboratory of the Philadelphia Zoological Garden. The animals were used for observations and as an occasional source of blood for clinical and immunologic studies. We visited the animals from time to time, often discussing our project with Dr. Robert Snyder, the director of the laboratory. Snyder had written his doctoral thesis on the sex ratio in the eastern woodchuck or groundhog (Marmota monax), basing his studies on observations of a large group of woodchucks in western Pennsylvania and, later, on a colony of the animals he kept at the zoo. He was aware of our interest in hepatitis and cancer of the liver and told us that these diseases occurred in wild and captive woodchucks. We tested the sera of some 40 of these animals for HBsAg using the immunodiffusion method and did not find any that reacted with anti-HBs.

In 1975 our colleague, Dr. Jesse Summers, began his work on the molecular biology of hepatitis B virus and discovered some of the unusual characteristics just described. As his work progressed, he commenced a search for other viruses that were similar to the hepatitis B virus, and we suggested that he test the woodchuck sera that had been used in the immunodiffusion testing. He screened the sera by centrifuging at high speed and assaying the pellets for endogenous DNA polymerase activity. He found that 15 per cent of the sera had particles that contained DNA polymerase. Three animals that had died of natural causes were studied in detail, two with hepatocellular carcinoma and one apparently of a myocardial infarction. The two animals with cancer had the DNA polymerase particles, whereas the animal that died of heart disease did not. The sera containing the DNA polymerase were studied by cesium chloride equilibrium centrifugation and other means. They contained a large number of spherical and tubular particles similar in size and structure to the corresponding HBsAg particles of hepatitis B virus. In the fractions containing the major DNA polymerase activity, larger particles (about 45 nm.) were found that closely resembled the large DNA-containing (Dane) particles of hepatitis B virus. The woodchuck particle DNA was about 3300 nucleotides in length, slightly larger than hepatitis B virus DNA.

The DNA has both single and double stranded regions. The single strand varied from virus to virus and was about as large as the equivalent region in the hepatitis B virus DNA. The nucleic acid homology between hepatitis B virus and woodchuck hepatitis virus was measured by liquid hybridization, and it was estimated that 3 to 5 per cent of the genomes (about 100 to 150 base pairs) were homologous.

Galibert et al. have come to a different conclusion using other methods. They compared the section of the hepatitis B virus DNA that codes for HBsAg with the equivalent section of the woodchuck hepatitis virus DNA and found that 487 of 678 residues were identical; that is, for this portion of the genome the homology is 74 per cent. It is likely that other portions of the molecule also have a great degree of homology.

There are remarkable similarities between the viruses. There are differences, however. Werner and her colleagues, using the reverse passive hemagglutination inhibition method, found that about 0.1 to 1 per cent of the surface antigen (HBsAg and WHsAg) is shared in common. This would account for the failure to find any reactivity in the earlier studies when the insensitive immunodiffusion method was used to search for hepatitis B virus-like viruses in the woodchuck sera. It is likely that there is more cross reactivity on the surface antigen than initially found. Millman has shown that radiolabeled anti-HBs can detect sera containing WHsAg. From Galibert’s study it can be predicted that there are major similarities between the surface antigen polypeptides of the respective viruses.

There was greater cross antigenicity between the core antigens of hepatitis B virus and woodchuck hepatitis viruses. About 5 to 10 per cent of mutual antigenic sites were found using crossover electrophoresis.

Cummings et al. cloned the hepatitis B virus DNA and then the woodchuck hepatitis virus DNA in the vector λgt WES, and these were subcloned into the kanamycin resistant plasmid pAO1. The clone produced a large amount of recombinant DNA of each of these viruses. They recalculated the size of the DNA as 3200 base pairs for the woodchuck hepatitis virus and 3150 base pairs for the hepatitis B virus. Tissue tropism of woodchuck hepatitis virus was de-
terminated by assaying the organs of five woodchucks with primary hepatocellular carcinoma for woodchuck hepatitis virus DNA.4 In every animal most of the activity was in the liver and the hemoplasma and very little in the other organs studied (kidney, lymph nodes, spleen, salivary glands, lung).

They also wanted to determine whether viral DNA is incorporated into the DNA of the cancer cells of the diseased liver and found that in two of the animals the results were consistent with the presence of one or two woodchuck hepatitis virus genomes integrated into the DNA of most of the cells and also that the integration occurred at the same site in all the cells. This finding, as the authors stated, is of major importance in evaluating the role of Woodchuck hepatitis virus (and by analogy hepatitis B virus) in the pathogenesis of primary cancer of the liver. The integration of the genome at a particular site in each of the tumor cells implies that the tumor originated from a single cell that had been infected with the woodchuck hepatitis virus and that the infection of the cell could have occurred at the time of, or before (but not after), the event that precipitated tumor formation.

POPULATION STUDIES OF WOODCHUCK HEPATITIS VIRUS

Tyler and her coworkers8 tested the sera of animals collected in locations in Maryland, New Jersey, and Pennsylvania within 220 km. of Philadelphia. In one collection 24 per cent of 166 animals had WHsAg (detected by passive hemagglutination inhibition using anti-WHs), and 42 per cent had anti-WHs (by passive hemagglutination). The frequency of infection and of carriers was about the same in males and females. However, there was a higher infection rate and a higher frequency of carriers in the older (age more than about 10 months) than in the younger animals. The authors suggested that this difference was due to the greater exposure to infection in the older animals and the possibility that host response changes with age.

The occurrence of primary hepatocellular carcinoma in wild caught (or killed) animals and in animals maintained in the colony at the Philadelphia Zoological Garden compared by age and longevity has been investigated by Snyder.9 Animals living in the protected environment of the zoo have a longer life expectancy than those exposed to the hazards of natural living. (This apparently is true for many species of animals.) Snyder, on the basis of release and recapture studies in wild animals, estimates that the mean age of death in free living animals was 14.9 months, and 50 per cent survived only eight months. The mean age of death of the zoo animals was 55.3 months, with 50 per cent surviving 56 months. About 25 per cent of the colony animals died with, and presumably of, primary hepatocellular carcinoma, the most frequent cause of death. The mean age of death was 63 months, and none of the captive animals died of cancer before the age of 23 months. The earliest case of primary hepatocellular carcinoma observed was in an animal that was significantly older than the mean age at death of the free living animals. Only 15 per cent of the free living animals survived until 23 months and less than 10 per cent until 60 months. Hence, very few animals in the wild would be expected to enter the time period when the risk of primary hepatocellular carcinoma is greatly increased. Snyder found four cases of primary hepatocellular carcinoma in 1007 autopsies performed on animals shot in the wild. He suggested that if a “correction” is made for the much younger average age of the wild animals, the frequency is similar to that of the zoo raised group. It has been suggested that the extraordinarily high frequency of primary hepatocellular carcinoma in the captive animals is a consequence of some carcinogenic factor within the colony area, but it is more likely to be due to the longer life expectancy of zoo animals, which allows them to enter the high risk age period. In nature, animals usually succumb for other reasons before they die of primary hepatocellular carcinoma—a common but not wholesome method of disease prevention.10

TRANSMISSION OF WOODCHUCK HEPATITIS VIRUS

Six young (four to eight month) woodchucks without signs of woodchuck hepatitis virus infection were injected with dilutions of serum containing woodchuck hepatitis virus.11 All the animals showed evidence of infection (WHsAg, anti-WHs, anti-WHc), but none was persistently infected. The incubation period increased with a decreasing concentration of inoculum, and the titers of virus that developed in the animals receiving the smaller inoculations were lower. As a result of this experience it appears that the woodchucks are good animals for studying infectivity of woodchuck hepatitis virus (and possibly hepatitis B virus). They may also provide a means for determining the factors that lead to chronicity following infection.

STRATEGY FOR FINDING ADDITIONAL ICRRNS

Following discovery of the woodchuck hepatitis virus it became feasible to set up a description of an icron, equivalent to the “type specimen” used by zoologists and botanists, to which the newly discovered virus could be compared.12 As new characteristics of the original and newly discovered viruses are uncovered, they can be added to the list of Icrons (Table 1). In due course a judgment can be made as to which characteristics are universal (for example, some of the features of the DNA) and whether subgroups of Icrons exist. It also suggests characteristics that can be used to decide which animals, organs, and tissues should be examined to find new viruses. The identification of woodchuck hepatitis virus in a Marmota suggested that other Sciuridae and Rodentia (the family and order in which Marmota is placed) should be examined (e.g., ground squirrels; see following
TABLE 1. CHARACTERISTICS OF ICRONS

<table>
<thead>
<tr>
<th>Particles</th>
<th>Hepatitis B Virus</th>
<th>Woodchuck Hepatitis Virus</th>
<th>Ground Squirrel Hepatitis Virus</th>
<th>Duck Hepatitis B Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present in large quantities in blood</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Virion structure double shell</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Virion diameter, nm.</td>
<td>40-45</td>
<td>40-45</td>
<td>47</td>
<td>40</td>
</tr>
<tr>
<td>Surface antigen particle, nm.</td>
<td>20-25</td>
<td>20-25</td>
<td>18-20</td>
<td>35-60</td>
</tr>
<tr>
<td>Elongated surface antigen, length, nm.</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;750</td>
<td>Not seen</td>
</tr>
<tr>
<td>Core, nm.</td>
<td>27</td>
<td>27</td>
<td>-27</td>
<td>27</td>
</tr>
<tr>
<td>Tryptophan/tyrosine ratios</td>
<td>High</td>
<td>?</td>
<td>High</td>
<td>?</td>
</tr>
<tr>
<td>DNA</td>
<td>Circular</td>
<td>Double and single stranded</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D/N polymerase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nucleotides (number)</td>
<td>3150</td>
<td>3200</td>
<td>3200</td>
<td>3000</td>
</tr>
<tr>
<td>Homology with hepatitis B virus, %</td>
<td>100</td>
<td>Yes</td>
<td>Yes</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>EcoRI sites (number)</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Antigens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface (hepatitis B virus, cross reacting %)</td>
<td>HBsAg</td>
<td>WHsAg(0.1-1)</td>
<td>GSHsAg(large)</td>
<td>DHBsAg</td>
</tr>
<tr>
<td>Core (hepatitis B virus, cross reacting %)</td>
<td>HBCAg</td>
<td>WHcAg(5-10)</td>
<td>GSHcAg(large)</td>
<td>DHBcAg</td>
</tr>
<tr>
<td>e</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Surface subtypes</td>
<td>Yes</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Responses to infection</td>
<td>Carrier state, % in population</td>
<td>0.1-20</td>
<td>0-20</td>
<td>0-50</td>
</tr>
<tr>
<td>Antisurface antigen, % in population</td>
<td>Anti-HBs</td>
<td>Anti-WHs</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Anticore antigen, % in population</td>
<td>Anti-Hbc</td>
<td>Anti-WHc</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Anti-e antigen</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Tropism</td>
<td>Liver</td>
<td>Liver</td>
<td>Liver, pancreas (?)</td>
<td></td>
</tr>
<tr>
<td>Clinical conditions</td>
<td>Acute hepatitis</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Primary hepatic carcinoma</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

section). The hepatitis B virus is found in primary cancer of the liver and has a tropism for liver. Other animals that develop cancer of the liver or chronic liver disease (e.g., certain ducks in China; see p. 1112) are therefore important candidates for investigation.

A VIRUS IN THE BEECHEY GROUND SQUIRREL

The Beechey ground squirrel (Spermophilus beecheyi) is a common mammal on the campus of Stanford University in California and a member of the Sciuridae family, as are the Marmota. Marion, Robinson, and their colleagues found that the sera of many of these animals had HBsAg reactivity by solid phase radioimmunoassay. All the sera with reactivity contained virus-like particles with DNA polymerase activity. They designated the virus as the ground squirrel hepatitis virus, by analogy with hepatitis B virus, even though liver tropism was not shown. (Dead animals were not available for testing.) The particles were very similar to the characteristic particles of hepatitis B virus and woodchuck hepatitis virus, with some interesting differences. The elongated particles were more numerous and their average length was significantly greater than that of other Icrons. There were also more large virion particles, and essentially all the sera containing particles also had DNA polymerase, whereas in the hepatitis B virus only a portion of HBsAg reactive sera has polymerase activity. The whole virion is slightly smaller than the hepatitis B virus. The DNA is very similar in size and structure, with the important difference that ground squirrel hepatitis virus DNA has two EcoRI cleavage sites, whereas hepatitis B virus DNA and woodchuck hepatitis virus DNA have only a single site.

There is a significant amount of cross reactivity between the surface antigens of hepatitis B virus and ground squirrel hepatitis virus. Appropriate anti-HBs antiserum can detect essentially all sera that contain ground squirrel hepatitis virus. This implies that there must be closely related configurations of the surface antigen polypeptides. A particular anti-HBs antiserum could be used to show two antigenic forms of ground squirrel hepatitis surface antigen GSHsAg, although it did not show heterogeneity in HBsAg.

HBsAg has two polypeptide chains measuring 25,000 and 29,000 daltons. They have nearly identical peptide compositions and similar primary sequences, and all the electrophoretic differences can probably be accounted for by the carbohydrate present on the larger but absent from the smaller polypeptide. GSHsAg has two polypeptides of 23,000 and 27,000 daltons, each slightly smaller than the equivalent
HBsAg polypeptides. They also have nearly identical peptide compositions. Gerlich et al.\(^\text{14}\) suggest that the larger polypeptide may differ from the smaller by a carbohydrate portion. It can be estimated that about 10 per cent less DNA sequence would be required for the 23,000 dalton GSHsAg polypeptide than for the equivalent 25,000 dalton HBsAg piece.

About one-third of the peptide spots of the 23,000 (P23) GSHsAg polypeptide are shared in common with the equivalent 25,000 (P25) HBsAg polypeptide, and about one-half of the spots of HBsAg P25 are shared with P23 polypeptide of GSHsAg. Gerlich et al. cite the as yet unpublished finding that the ground squirrel hepatitis virus core antigens (GSHcAg) have more cross reactivity with HBcAg than do the respective surface antigens. This greater variability in surface antigen than in core antigens is also a feature of many other enveloped viruses. It generates the interesting concept that for a given genome, viruses maintain a greater variability on the surface than internally, perhaps as a primary mechanism to restrict host species specificity and to allow for variation of immunologic response with the members of the host species.

**DUCK HEPATITIS B VIRUS**

In 1977, while in the northern Kwangsi Autonomous Region, an area of high endemicity for primary hepatocellular carcinoma, I was told that in some regions of the People’s Republic of China, where primary hepatocellular carcinoma is common in humans, it is also common in domesticated ducks. The following year, in collaboration with Dr. T.-T. Sun of Beijing, sera were obtained from farmyard ducks collected in Chi-tung County on the north bank of the Yangtze across from Shanghai City. A virus similar in appearance to hepatitis B virus was seen in the sera of 11 of the 33 ducks.\(^\text{15}\) A series of investigations was started on these particles.

Mason attempted to infect domesticated Pekin ducks (*Anas domesticus*) obtained from commercial breeders in the United States with this presumed virus. In doing so, he found that the Pekin ducks were already infected with a virus similar to the hepatitis B virus and to the virus found in their Chinese cousins. (Pekin ducks were imported to America from China in the nineteenth century.) He and his colleagues found that four of 12 Pekin ducks tested had DNA polymerase similar to that found in the hepatitis B virus and the woodchuck hepatitis virus. These sera contained virus-like particles about 40 nm in diameter, similar in appearance to the large virion particles of the other icrons. Core particles could be distinguished in the whole and disrupted virions, and these were similar in appearance and size to the core particles of the other viruses.\(^\text{16}\)

The nucleic acid was circular, double and single stranded, and about the same size as that of the other viruses. Particles that appeared to be analogous to the surface antigen particles of hepatitis B virus were more heterogeneous in size and shape than the equivalent particles in hepatitis B virus, woodchuck hepatitis virus, and ground squirrel hepatitis virus. They estimated hepatitis B virus and duck hepatitis B virus (DHBV, as they called it) to have less than 10 per cent DNA homology. In analysis of the tissue of one infected animal, using an annealing rate assay for viral DNA, they found most of the activity in the liver. A much smaller amount was found in the pancreas, but it was significantly greater than in the other organs tested. Of 219 ducks of different ages and sex, 26 (12 per cent) had duck hepatitis B virus, and it was present about equally in both sexes and in all ages, from one day to 14 months. O’Connell and London\(^\text{17}\) found virus in embryos removed as early as 15 days after the eggs were laid. This strongly suggests that infection of the embryo by the mother is an important mechanism for virus transmission. Maternal infection may be important in many human populations, and the duck hepatitis B virus studies would permit interesting cross species comparisons.

**DISCUSSION**

There are several properties present in all icrons (Table 1). The “carrier” state (that is, the presence of large amounts of virus or surface antigen particles in the peripheral blood without apparent illness) occurs with a high frequency in one or more of the populations of the affected species. The virion and core structure and size are similar. The DNA shape (relaxed circular), size (3000 or so nucleotide pairs), double and single strandedness, and the presence of DNA polymerase in the core are additional universal characteristics. The ground squirrel hepatitis virus has two EcoRI sites that distinguish it from the hepatitis B virus, woodchuck hepatitis virus, and duck hepatitis B virus, which have only one.\(^\text{17a}\) There has been a considerable amount of homology in the nucleotides and in the surface antigens when they have been compared. The cross antigenicity appears to be greater for the core than for the surface antigen, suggesting that the outside covering is in a position to preserve its antigenic separateness, which should decrease the probability of cross species infection. There is also the potential that under some natural or other conditions, this separateness could be altered, since many peptide and nucleotide sequence similarities exist between the surface antigens and their genes.

It is not known whether the viruses from different species can cross infect individuals from other species and, if so, whether they cause disease. Epidemiologic and clinical studies can be done very rapidly when strong antisera and antigen preparations are available for serologic and organ testing.

Liver tropism is characteristic of icrons in the animals in which it has been studied. The duck hepatitis B and ground squirrel hepatitis viruses were initially found in carrier animals that were not apparently ill, and this may be the case for icrons discovered in the future. A study of the distribution of
the virus in the tissues and organs of the carriers would predict which organs would be affected in the host that develops disease. By analogy, in human carriers the hepatitis B virus is concentrated in the liver, and this indicates the organ involved in individuals who become sick after infection. The relatively small amount of duck hepatitis B virus in the pancreas suggests that icrons not yet studied or discovered might be related to chronic diseases of this organ.

From where did these viruses arise? How long have they existed in the species they currently infect, and were they transmitted to other species during the course of evolution? Would it be possible to identify an animal genome whence this virus arose, if in fact that was its beginning? What was the "primordial" virus?

Recent observations and experiments relating to speciation could generate interesting speculation and studies of hepatitis B virus-like viruses in different species. According to this approach, speciation could in some cases result from the isolation of a few members of a widespread population, and these founding members could have a very different genetic inventory from that of most of the members of the parent population. This is thought to arise as a consequence of chance, but other mechanisms for markedly changing the genetic makeup of the founding members might also occur; for example, the introduction of "new" genes into the host genome by viral infection. Hence, subsequent generations would be affected by widespread infection in parents or ancestors, and this could provide the genetic differences postulated for this mechanism of species evolution. It should become possible to address and possibly answer some of these questions as we learn more about icrons and their distribution in the natural world.

There is now substantial evidence that persistent hepatitis B virus infection is required for the development of primary cancer of the liver (see reference 11 and the article by London on page 1085). The vaccine introduced by Blumberg and Millman has now successfully been tested by Szmuness and his colleagues. The imminent availability of the vaccine has accelerated planning for studies to prevent primary cancer of the liver, one of the most common and most deadly cancers in the world. The discovery of a relation between a second icron (woodchuck hepatitis virus) and possibly a third (duck hepatitis B virus) to cancer of the liver in the species they infect raises the expectation that one can identify other virus-cancer relations that might also be preventable, and much of our current research is directed to that end.

REFERENCES


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Philadelphia, Pennsylvania 19111
Hepatitis B surface antigen and polymerized albumin binding activity in sheep serum

(anti-hepatitis B surface antigen/hepatitis B binding substance/anti-polymerized albumin/Ausab assay/IgM)

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Contributed by Baruch S. Blumberg, September 22, 1983

ABSTRACT Sera from sheep and other domestic animals contain a substance that gives a strongly positive test for antibody to hepatitis B virus surface antigen by the accepted radioimmunoassay procedure. We have purified this substance from sheep serum to near homogeneity by ion-exchange, affinity, and molecular exclusion chromatography and have identified it to be an IgM. We present evidence that this sheep IgM is an antibody to polymerized sheep albumin. This antibody may arise due to infection by hepatitis B virus, hepatitis B virus-like viruses, or other pathological agents and may react with hepatitis B virus surface antigen by combining with polymerized albumin bound to the hepatitis B virus receptor for this polymer.

In an earlier report we described a glycoprotein, contained in the sera of a variety of animals, which has a high affinity for hepatitis B surface antigen (HBsAg) (1). This substance (termed “hepatitis B binding substance,” HBBS) gave a strongly positive test by the commonly used radioimmunoassay (RIA) for antibody to HBsAg (anti-HBsAg). The reaction between HBsAg and anti-HBsAg in the RIA could be totally inhibited by prior incubation of the HBBS with HBsAg. However, tests done at this time did not support the hypothesis that HBBS was an antibody to HBsAg. We were thus interested in learning the basis of this binding phenomenon.

We have now isolated this material from sheep serum and, contrary to the earlier conclusions, have identified it as an IgM antibody. We report here on the characterization of this IgM and present evidence consistent with the hypothesis that this IgM is an antibody to polymerized sheep albumin (PS albumin). As in humans (2–4), anti-PS albumin may arise in sheep due to replication in the liver of hepatitis B virus (HBV)-like or other viruses. Reaction with HBsAg may be due to infection by hepatitis B virus, hepatitis B virus-like viruses, or other pathological agents and may react with hepatitis B virus surface antigen by combining with polymerized albumin bound to the hepatitis B virus receptor for this polymer.

MATERIALS AND METHODS

Assay for HBsAg Binding Activity. During purification, anti-PS albumin (formerly HBBS) in serum samples and column fractions was detected by its affinity for HBsAg by using the Ausab solid-phase RIA for anti-HBsAg (Abbott). In this assay, plastic beads, coated with purified HBsAg, are exposed to serum or other sources of anti-HBsAg or HBsAg. The beads are then washed, exposed to 125I-labeled HBsAg (125I-HBsAg), washed again, and assayed for radioactivity. Activity was expressed as the ratio of cpm of test sample to mean cpm of normal human serum supplied by the manufacturer.

Animal Sera. Commercial lots of sheep sera were periodically screened for binding activity by the Ausab assay. The main sources were Miles (Pentex lot 432), and Dutchland Laboratories (Denver, PA; lot 82080-8C).

RESULTS

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Immunology: Franklin et al.

**Fig. 1** Equilibrium chromatography of sheep serum on DEAE-Sephadex A-50. —, % T_{280}; --, Ausab activity. Fractions were pooled as indicated. Buffer was 0.20 M sodium phosphate (pH 5.75) containing 0.02% sodium azide.

Fig. 1 shows the equilibrium chromatography of sheep serum on DEAE-Sephadex (Fig. 1). Anti-PS albumin (detected by its Ausab activity) was recovered as a major peak with a small shoulder, which eluted at the breakthrough peak of the column. The activity was well separated from the albumin peak (fraction 3). The material from fraction 1 of the DEAE-Sephadex column was applied to a Cibacron blue F3GA (Affi-Gel blue, Bio-Rad) column (Fig. 2), which was washed with the equilibration buffer until the breakthrough peak (which was devoid of activity) emerged. Then the same buffer containing 1.4 M NaCl was applied, which eluted the activity as a sharp peak corresponding to the leading edge of the material absorbing at 280 nm. The elution profile of fraction 2 from the DEAE-Sephadex column was similar to that of fraction 1 on Affi-Gel blue but, due to its low yield, was not further studied. As a final purification step, Affi-Gel blue fraction 2 was applied to a Sephacryl S-300 column that had been previously calibrated with molecular weight marker proteins (Fig. 3). The Ausab activity emerged at essentially the excluded volume of this column, having an apparent Mr of 800,000, suggestive of the presence of an IgM. The second peak eluting from this column was devoid of Ausab activity and had an apparent Mr of 150,000, which suggested that it consisted of IgG.

**IgM Nature of Anti-PS Albumin.** Each of the active fractions obtained throughout the purification procedure was analyzed by NaDodSO4/polyacrylamide gel electrophoresis (Fig. 4). Because the same quantity of protein was applied to each slot, only the dominant species of each fraction is seen. The starting serum shows principally bands of Mr's 67,000, 54,000, and 28,000, which most likely represent albumin and the heavy and light chains of IgG, respectively. The active fractions from the DEAE-Sephadex and Affi-Gel blue columns also show bands that comigrate with those of the IgG standard. However, the active material from the Sephacryl S-300 column shows bands of Mr's 74,000 and 28,000 that comigrate with those of the IgM standard, supporting the indications from the gel filtration experiments that the active material is IgM.

Immunodiffusion of the active fraction against anti-sheep IgM, IgG, albumin, and complement C3 confirmed the presence of IgM. Preincubation of the Ausab active material with anti-sheep IgM inhibited the activity by >90%, whereas anti-sheep IgG inhibited by <20%. Mild reduction (15 mM mercaptoethanol) and alkylation with iodoacetamide destroyed >90% of the Ausab activity, which indicated that the pentameric IgM structure is required for activity and that IgG does not play a significant role in generating this activity.

The purified IgM did not appear to be anti-HBsAg. We reported previously that immunodiffusion of HBBS against HBsAg did not produce a precipitin band (1). In further studies we find that preincubation of the IgM with 125I-HBsAg (Abbott) (in the presence or absence of unlabeled HBsAg carrier) failed to inhibit precipitation of HBsAg by anti-

**Fig. 2.** Chromatography of DEAE-Sephadex column fraction 1 on Affi-Gel blue. Symbols are as in Fig. 1. Elution was with 0.02 M sodium phosphate buffer (pH 7.1) containing 0.02% sodium azide. At the gap, elution with the starting buffer containing 1.4 M NaCl was initiated.

**Fig. 3.** Gel filtration of Affi-Gel blue column fraction 2 on Sephacryl S-300. Symbols are as in Fig. 1. Buffer was 0.02 M sodium phosphate (pH 7.1) containing 0.5 M NaCl and 0.02% sodium azide.

**Fig. 4.** NaDodSO4/polyacrylamide gel electrophoresis of Ausab-positive fractions. Ten micrograms of total protein was loaded per well. Lane 1, sheep serum; lane 2, DEAE-Sephadex fraction 1; lane 3, Affi-Gel blue fraction 2; lane 4, Sephacryl S-300 fraction 1; lane 5, sheep IgM; lane 6, sheep IgG; lane 7, molecular weight marker proteins. Stain was Coomassie brilliant blue.
Fig. 5. RIA for anti-PS albumin (PSA) activity. IgM (Sephacryl S-300 fraction 1); IgG (Sephacryl S-300 fraction 2); heterogeneous sheep serum proteins (Affi-Gel blue fraction 1); glycine.

HBsAg. Furthermore, if the IgM is preincubated with $^{125}$I-HBsAg, anti-sheep IgM fails to precipitate $^{125}$I-HBsAg, even upon the further addition of anti-sheep globulin. This suggests that an IgM-$^{125}$I-HBsAg complex does not form.

An IgM antibody in humans has been found to combine with PH albumin (2-4), which is probably bound to the PH albumin receptor on HBsAg (5). These findings and our data prompted us to test the hypothesis that the sheep IgM we isolated is anti-PS albumin.

Characterization of Anti-PS Albumin Activity. Immunodiffusion of the purified IgM against PS albumin ($M_r = 600,000$) produced a sharp, somewhat weak, precipitin band. No band was obtained with the modified monomer or intermediate molecular weight polymers. These preliminary results indicated that the IgM fraction contained antibodies against determinants unique to PS albumin.

Results of a typical RIA for anti-PS albumin activity are shown in Fig. 5. Both of the controls, which consisted of Sepharose-coupled glycine and fraction 1 from the Affi-Gel blue column (immunoglobulins and other serum proteins), showed very little tendency to bind PS albumin. The IgG fraction, which copurified with the IgM up to the last (Sephacryl S-300) step in the fractionation, shows some binding activity. Clearly, the highest affinity for PS albumin resides in the IgM. Addition of 1 mg of monomeric sheep albumin per 250-μl assay caused no inhibition of binding, whereas 16 μg of unlabeled PS albumin diminished binding by 62%, supporting the notion that the antigenic determinants do not reside on the monomeric albumin.

These binding data were subjected to Scatchard analysis (9), as shown in Fig. 6. No apparent binding existed at all for the glycine and serum protein controls. The IgG component did show binding with an apparent valence of 1.8, whereas the IgM had an apparent valence of about 6.0. The slope of the plot for both the IgG and IgM was essentially the same and gave an apparent affinity constant of $4.6 \times 10^9$ liters/mol.

**DISCUSSION**

The results reported here indicate that what we termed HBBS, originally detected by its affinity for HBsAg, is an IgM antibody to PS albumin and not to HBsAg. This affinity is probably not a general property of sheep IgM as the active material comprises only about 4% of the reported total IgM contained in sheep plasma (10). Monomeric sheep albumin does not prevent anti-PS albumin from combining with PS albumin. Thus, the antibody activity appears to be specific for determinants formed by the polymerization process in accordance with the human and murine data (2, 3) and to have a rather high affinity constant. The anti-PS albumin IgM had an apparent valence of 6. Although $10^{10}$ combining sites per mol of IgM are theoretically expected, interference often renders some antibody sites inaccessible to antigen in the case of large antigens such as PS albumin (11). Some IgM antibody to PS albumin also is produced and exhibits a valence of 1.8. It may be that certain combining site similarities cause it to copurify with the IgM up to the last fractionation step. The absence of a reaction between anti-PS albumin and commercial $^{125}$I-HBsAg may be due to removal of the polymerized albumin during the purification process. Reactivity of the anti-PS albumin with Ausab beads may be due to the presence of albumin, which is added as a blocking agent, mimicking its polymerized form when absorbed to the plastic beads. Alternatively, the antigen used for bead preparation may still have PH albumin bound to its receptor [antibodies to polymerized albumins crossreact between species (12)].

Antibodies to PH albumin ($M_r = 570,000$) have been found in the sera of patients having hepatitis and other liver diseases (2-4). HBsAg has a species specific receptor site for PH albumin of $M_r = 500,000$ (5). This site appears to reside in the 55-amino-acid peptide NH$_2$-terminal to HBsAg that is coded for by the pre-S region of the HBV genome (13). Complexes of IgM and HBsAg have been observed in the serum of patients with acute viral hepatitis (14). Persistence of these complexes for more than a month seems to be a reliable prognostic indicator for persistence of the carrier state. Blocking experiments with PH albumin suggest that the IgM component in these complexes is anti-PH albumin (15). Elu-
tion experiments on the complex failed to demonstrate that the IgM is anti-HBsAg (16). However, no direct tests of these notions have been performed.

Our results indicate that sheep also develop anti-polymerized albumin, perhaps initiated by a HBV-like or other pathological agent. Anti-PS albumin appears to mimic anti-HBsAg using the currently acceptable RIA for anti-HBsAg. Furthermore, in this paper we provide direct evidence that the IgM is an antibody to PS albumin. However, we have not detected viral markers in any ovine or bovine sera.

Hoofnagle et al. have confirmed our earlier survey on the ubiquity of this antibody and its reactivity with HBsAg (17). They also find the activity resides in an IgM. Because a booster injection of HBsAg caused an increase in titer of “anti-HBsAg” in rabbits that had preexisting antibody, these authors concluded that the IgM was an antibody to HBsAg. However, our evidence suggests that they were in fact studying anti-polymerized albumin. The reaction of anti-polymerized albumin with HBsAg most likely occurs through polymerized albumin bound to the receptor on the viral surface. Thus, a booster injection of HBsAg may also contain polymerized albumin. Anti-polymerized albumin isolated from animal sources may be useful in the production of diagnostic assays or in affinity purification of hepatitis-like viruses. The presence of polymerized albumin receptors on hepatocytes (18) may be responsible for viral tropism to the liver. Induction of anti-polymerized albumin in an animal model such as the woodchuck may modify hepatitis infectivity or the carrier state (or both).

We acknowledge technical assistance from Heidi Simmons and Theresa C. Halbherr. This work was supported by U.S. Public Health Service Grants CA-06551, RR-05539, and CA-06927 from the National Institutes of Health, by an appropriation from the Commonwealth of Pennsylvania, and by a grant from the Kaiser Family Foundation.


Lack of perinatal transmission of hepatitis B virus infection in Senegal, West Africa

Between 1977 and 1980, 1442 pregnant women in Thies, Senegal, were tested for serologic markers of hepatitis B virus (HBV) infection. Of these, 9.8% were HBsAg(+), 59.9% were anti-HBc(+), and 15.6% had anti-HBc alone. Of 116 HBsAg(+), pregnant women, only 19.8% were HBsAg(+), a much lower proportion of infectious carriers than seen in Asian populations. Cord blood from 1353 babies was HBsAg(−), implying that the babies were not infected prior to birth. Four hundred sixty-two babies, including 88 born to HBsAg(+) mothers, were observed for 2 weeks to 38 months after birth. In contrast to observations in Asia, none of the babies became HBsAg(+) before 5 months of age, and only three of the 16 born to HBsAg(+) mothers became HBsAg(+) within the first year of life; all three developed chronic infections (i.e., HBsAg(+) for ≥6 months. In the second year of life, six of 34 babies born to HBsAg(+), HBsAg(−)/anti-HBc(−) mothers became infected with HBV, and four of the six developed chronic infections. During the first 3 years of life, infections occurred at a higher rate in infants born to HBsAg(+), HBsAg(−)/anti-HBc(−) mothers than in HBsAg(−), women. The latter group of infants included 4.0% of those born to anti-HBc(+) mothers, 4.6% born to anti-HBcAg(+), and 3.2% born to uninfected women. These observations indicate that HBV infections in Senegal usually do not occur perinatally, but do occur at high incidence later in infancy and childhood. Such infections can be prevented by the use of hepatitis B vaccine alone; administration of hepatitis B immune globulin should not be needed. (J Pediatr 106:843, 1985)

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Paris, France, Philadelphia, Pennsylvania, and Dakar, Senegal, West Africa

Hepatitis B virus infection and chronic carriers of the virus are common in many tropical countries, particularly Asia and sub-Saharan Africa. A high incidence of infection may also occur in Arctic communities. There is now substantial evidence (recently reviewed) that chronic infection with HBV is etiologically associated with the development of primary hepatocellular carcinoma. In areas endemic for HBV, PHC is the most common or one of the most common malignant tumors. In a study in Taiwan of chronic HBV carriers and controls, the relative risk of PHC in carriers was >200 times that of noncarriers. More than 95% of cases of PHC have developed in chronic carriers.

See related article, p. 777.

| HBV | Hepatitis B virus |
| PHC | Primary hepatocellular carcinoma |

In a study in Senegal, we found that mothers of patients with PHC were more likely to be carriers (70%) than mothers of matched individuals who did not have PHC (10%). This finding was subsequently confirmed in Korea and in Taiwan. From this observation we inferred that many persons who eventually develop PHC have been infected by their mothers early in life.
Table I. Number of children's sera collected at different ages according to mother’s HBV response

<table>
<thead>
<tr>
<th>Status of mothers</th>
<th>Age of babies</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 wk to 3 mo</td>
<td>3 to 5 mo</td>
<td>5 to 12 mo</td>
<td>1 to 2 yr</td>
<td>2 to 3 yr</td>
</tr>
<tr>
<td>HBsAg(+)</td>
<td>26</td>
<td>16</td>
<td>40</td>
<td>42</td>
<td>18</td>
</tr>
<tr>
<td>Anti-HBsAg(+)</td>
<td>153</td>
<td>55</td>
<td>94</td>
<td>52</td>
<td>26</td>
</tr>
<tr>
<td>Anti-HBeAg(+) only</td>
<td>42</td>
<td>13</td>
<td>28</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Negative</td>
<td>36</td>
<td>12</td>
<td>27</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>257</td>
<td>96</td>
<td>189</td>
<td>114</td>
<td>55</td>
</tr>
</tbody>
</table>

Some babies are represented more than once if specimens were obtained from same child at different ages; 462 babies are included.

Table II. Effect of mothers' HBV status on babies' HBV status at birth

<table>
<thead>
<tr>
<th>HBV status (cord blood) of babies</th>
<th>HBsAg(+)</th>
<th>Anti-HBsAg(+)</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg(+)</td>
<td>0*</td>
<td>0*</td>
<td>0</td>
<td>0*</td>
</tr>
<tr>
<td>Anti-HBsAg(+)</td>
<td>8</td>
<td>108</td>
<td>376</td>
<td>745</td>
</tr>
<tr>
<td>Negative</td>
<td>124</td>
<td>815</td>
<td>406</td>
<td>1353</td>
</tr>
<tr>
<td>Total</td>
<td>132</td>
<td>815</td>
<td>406</td>
<td>1353</td>
</tr>
</tbody>
</table>

Association is highly significant; P < 0.0001.

*At beginning of study, cord blood sera of seven babies were found to be HBsAg(+) This apparently was caused by contamination of baby's cord blood sample with maternal blood. Procedures for collection of cord blood were revised and carefully followed. No positive cord blood sera were detected thereafter, and these seven mother-baby pairs were excluded.

Studies in Asia, Africa, and elsewhere have shown that maternal transmission plays a large role in transmission of HBV and in the development of the carrier state. Maternal transmission, however, does not necessarily mean that infection takes place in the prenatal period. Carrier mothers who are also HBsAg(+) are more likely to transmit HBV to their newborn infants than those who are anti-HBsAg(+) or do not have either HBsAg marker, but little is known about this relation in African families.

Infection at an early age, particularly maternally transmitted infection, may increase the risk of developing chronic liver disease and PHC, and decrease the age of onset. Hence, the mother-child relationship in respect to HBV infection is very important in understanding the pathogenesis of chronic liver disease and PHC, and in developing methods for the control of infection, particularly use of the newly available hepatitis B vaccine. We report a prospective study of mother-child-HBV relationships conducted in Senegal, West Africa.

METHODS

Blood specimens were obtained from mothers visiting a mother-child clinic and from their offspring at about the time of birth and at periods up to 3 years after delivery. The clinic is in Thies, the second largest urban center in Senegal, located 80 km east of Dakar, the capital. About 7000 deliveries per year are performed in the clinic in-patient facility. Between April 1977 and April 1980, 1442 women admitted for delivery were interviewed, a questionnaire completed, and specimens of maternal and cord blood obtained. Only time and personnel available determined which women were selected for study. The information obtained included name, address, ethnicity, age, and previous history of jaundice, and birth date, birth weight, and sex of the offspring.

Maternal sera were collected and immediately tested for HBsAg at the Centre National de la Transfusion Sanguine in Dakar by reverse passive hemagglutination. All babies born to HBsAg(+) mothers were included in the follow-up. Follow-up was also conducted in HBsAg(−) mothers and their offspring. The intention was to choose as controls the next two HBsAg(−) mothers who lived in the same district as the HBsAg(+) women. As the study proceeded, many babies could not be followed, and we ultimately accepted as controls all the mothers who agreed to keep their babies in the study after the first (15 day) follow-up visit, regardless of the district. Four times as many HBsAg(−) as HBsAg(+) women were studied. Controls came from all districts where HBsAg(−) women lived. Blood collections from the children were planned for intervals of approximately 15 days; 3, 6, and 12 months; and then yearly for up to 3 years; and from the mothers at 6 months after delivery (Table I). Most babies submitted two or more follow-up samples; in some, all the planned samples were collected.

All sera, including those tested in Dakar, were tested at the Institute for Cancer Research in Philadelphia for HBsAg by radioimmunoassay (Austria II; Abbott Labora-
Anti-HB, was determined by passive hemagglutination against red blood cells coated with HB,Ag subtypes ad and ay, separately, and were scored as positive if titers were ≥1:4 and could be inhibited by HB,Ag(+) serum (Electro-Nucleonics, Bethesda, Md.). Titers of 1:4 and 1:8 were categorized as low; ≥1:16 but <1:128 as medium; and ≥1:128 as high. Anti-HB, and HB,Ag were determined by RIA (Corab; Abbott Laboratories) (Abbott Laboratories). Anti-HB, was only tested by a micro-micro immunodiffusion method developed in our laboratory (personal communication). All sera were assayed for HB,Ag and anti-HB. Those negative for both were then tested for anti-HB,Ag. One hundred sixteen of the mothers' sera positive for HB,Ag were tested for HB,Ag and anti-HBeAg.

The "survival" analyses were done by the method of Kaplan and Meier.

RESULTS

Mothers and children studied. A total of 462 babies (242 boys), including 88 born to HBsAg(-) mothers, were observed for 2 weeks to 38 months. One to six blood specimens were obtained for each baby (Table I). Babies born to 88 HBsAg(-) and 374 HBsAg(--) women were observed. Ten infant deaths were reported during the perinatal period (six babies of carriers and four born to HBsAg(--) women); the cause of death was not recorded.

A total of 1442 pregnant women were tested. Of these, 141 (9.8%) were HBsAg(+), 864 (59.9%) had anti-HB, and 225 (15.6%) had anti-HB, alone. Only 212 (14.7%) did not have any evidence of infection. The responses did not differ significantly by ethnic group. Total rate of HBV infection increased with age (P <0.0016), but the prevalence of HB,Ag remained about the same (Fig. 1). Only one case of clinical liver disease was found; a woman who was HBsAg(-) on first testing had PHC and died 2 months after the delivery of her child.

The presence of HB,Ag and anti-HB, were determined in 116 of the mothers who were HBsAg(+). Of these, 23 (19.8%) were HBsAg(+), 24 (20.7%) were anti-HB,(+), and the remaining 69 (59.5%) had neither. Because HB,Ag was tested by RIA, but anti-HB, was tested by the micro-micro immunodiffusion method, the high proportion of HB,Ag(--) mothers probably reflects the relative insensitivity of the micro-micro immunodiffusion method.

Findings at birth. There were 1353 pairs of mothers and their newborn babies, but the questionnaire information was not complete for all pairs. Cord blood samples of all babies were HBsAg(-), implying absence of in utero infection (Table II). There were 815 mothers who had anti-HB, and 707 (86%) of their offspring also had anti-HB, (Table II). Further, the titers of anti-HB, in mothers and their children were similar (Table III); 531 of the children and mothers had about the same titer. For the remaining 176, the children's antibody titers were higher or lower than the mothers', but in most instances the differences were equal to or less than twofold, which could be accounted for by variations in the assay system. Thirty children with HBsAg(--) mothers had anti-HB, (Table II). In all 30, the anti-HB, titers were low (≤1:16), suggesting to us that anti-HB, below the level of detectability was probably present in the maternal sera.

Events after birth. Babies who were anti-HB,(+) at
Table IV. Transient* and chronic† infections in children at different times after birth according to HBV status of mother

<table>
<thead>
<tr>
<th>Age of babies</th>
<th>Infection events</th>
<th>HBV status of mothers</th>
<th>Noncarrier</th>
<th>Carrier</th>
<th>Total</th>
<th>Cumulative total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Uninfected‡</td>
<td>anti-HB,(+)$</td>
<td>anti-HB(+)</td>
<td>HB,Ag(+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 wk to 5 mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transient</td>
<td>Noncarrier</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>Noncarrier</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carrier</td>
<td>62</td>
<td>65</td>
<td>247</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total sera collected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 to 12 mo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transient</td>
<td>Noncarrier</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>Noncarrier</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carrier</td>
<td>33</td>
<td>30</td>
<td>123</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total sera collected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 to 24 mo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transient</td>
<td>Noncarrier</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>Noncarrier</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carrier</td>
<td>14</td>
<td>15</td>
<td>68</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total sera collected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 to 38 mo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transient</td>
<td>Noncarrier</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>Noncarrier</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carrier</td>
<td>5</td>
<td>5</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total sera collected</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>62</td>
<td>65</td>
<td>247</td>
<td>88</td>
</tr>
</tbody>
</table>

In each time period, only new events are recorded, and babies infected are not counted in next period. Total collected, number of babies at risk of HBV infection collected during indicated period.

*Child anti-HB(−) at birth, and subsequently anti-HB,(+) at titer ≥1:4 or anti-HB,(+) after HBsAg(+) test. Babies anti-HB,(+) at birth who had twofold increases in titer in two subsequent samples or who became anti-HB,(+) after two anti-HB,(−) tests.
†Child became HB,Ag(+), and remained positive on all subsequent tests or on last sample tested.
‡HBsAg(−), anti-HB(−), anti-HBe(−).
§Anti-HB(−), HB,Ag(−).

birth lost this antibody within 6 to 9 months of age (Table IV). As might be expected, the children with high titers were slowest to lose antibody.

There were no infections prior to 5 months of age, and this was independent of the mothers’ response category.

During the follow-up periods (maximum 38 months), 30 infections (eight transient and 22 chronic) occurred. The carrier mothers had children with the highest frequency of both chronic and transient infection events. Seventeen percent of the offspring of carrier mothers became infected, whereas only 4% were infected in the other categories (P <0.0001).

The children of mothers with any marker became infected earlier and in higher frequency than the offspring of mothers who were uninfected (Fig. 2). Most of the infections occurred in the children of carrier mothers. In the mothers who were anti-HB(−), children did not become infected until 9 months of age. (The only exception was probably a laboratory error, in a child who had a high titer of anti-HB, in cord blood was anti-HB(−) at 15 days, and developed a low titer of anti-HB, at 5 months of age.) HB,Ag and anti-HB, were determined in 87 HBsAg(+) mothers whose babies returned for follow-up. The offspring of the HB,Ag(+) mothers became infected before the children of carrier mothers with neither HB,Ag nor anti-HB, and the offspring of carriers with anti-HB, were the last to become infected. None of 16 babies born to HB,Ag(−) women became HB,Ag(+) before 5 months of age. By the end of the 38 months, 25% of the babies born to HB,Ag(+) mothers had become infected, and all of these were chronic infections (Table V). The anti-HB,(−) and HB,Ag(−)/anti-HB(−) mothers had fewer chronically infected babies than the HB,Ag(+) mothers, but more than HB,Ag(−) mothers (8% compared with 4%).

DISCUSSION

The frequency of HBV markers in our study population of mothers is similar to that found in other African females.15,13,24,25 The frequency is lower than in Taiwan19 but higher than in Japan11 and in western countries.15,17,26,27
Fig. 2. Survival curves showing percentage of children remaining uninfected at successive time intervals after birth according to HBV status of mothers. Infected mothers had more infected offspring than did uninfected mothers, and most infections occurred in offspring of carrier mothers. ▲, Babies born to HBsAg(+) mothers (83.0% uninfected at 35 months); X, babies born to anti-HB, (+) mothers (95.4% uninfected at 35 months); △, babies born to anti-HB(+) mothers (95.6% uninfected at 35 months); ●, babies born to uninfected mothers (96.8% uninfected at 35 months).

Table V. Infection of offspring of carrier (HBsAg(+)) mothers according to time after birth and HBsAg, anti-HB, response of mothers

| Age of babies | Infection events | e status of HBsAg(+) mothers | Cumulative total | Total |
|---------------|------------------|-------------------------------|-----------------|
|               |                  | HBsAg(+) | anti-HB (+) | HBsAg(+), anti-HB(−) |                   |
|               |                  | n | % | n | % | n | % | n | % |
| 1 wk to 5 mo  | Transient        | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|               | Chronic          | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|               | Risk(n)          | 16| 20| 51 | 87 | 0 | 0 | 87 | 87 |
| 5 to 12 mo    | Transient        | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
|               | Chronic          | 3 | 0 | 0 | 3 | 0 | 0 | 3 | 3 |
|               | Risk(n)          | 15| 15| 47 | 77 | 0 | 0 | 77 | 87 |
| 12 to 24 mo   | Transient        | 0 | 0 | 2 | 2 | 4 | 4 | 7 | 7 |
|               | Chronic          | 0 | 0 | 4 | 4 | 0 | 0 | 4 | 4 |
|               | Risk(n)          | 8 | 12| 34 | 54 | 0 | 0 | 54 | 87 |
| >24 mo        | Transient        | 0 | 2 | 2 | 2 | 4 | 4 | 4 | 4 |
|               | Chronic          | 1 | 1 | 1 | 1 | 3 | 3 | 3 | 3 |
|               | Risk(n)          | 3 | 5 | 8 | 8 | 16| 16| 16| 16|
| Total         | Transient        | 4 | 25.0| 2 | 10.0| 2 | 3.9| 4 | 4.6|
|               | Chronic          | 1 | 5.0 | 5 | 9.8 | 10 | 11.5|
|               | Transient + chronic | 4 | 25.0| 3 | 15.0| 7 | 13.7| 14 | 11.1|
|               | Risk(n)          | 16| 20| 51 | 87 | 0 | 0 | 87 | 87 |

The presence of HBsAg in carriers (an indicator of infectivity) is significantly lower in our African study population than in populations from the Far East.18, 19, 28 There are several possible explanations for these differences.

First, there may be differences in the sensitivity of the technique we used for measuring HBsAg and that of other investigators. That is, we may not have detected some significant number of HBsAg(+) sera. However, this is unlikely, because the commercial RIA (Abbott Laborato-
ries) is a highly sensitive, reliable test. Moreover, the low rate of transmission of HBV from HBsAg(+), HBsAg(−) mothers to their babies is consistent with the assay for HBsAg being accurate.

Second, HBsAg is associated with higher HBsAg titer, viral DNA polymerase, HBV DNA, and probably the amount of whole virus produced. Therefore, infected Africans may produce less virus than Asians and others. If this is true, then there may be geographic or genetic differences in virus cell interactions.

The offspring of carrier mothers were more likely to become infected than the children of the other mothers; this confirms previous studies in Senegal and elsewhere. Many of the offspring of non-HBsAg carrier mothers also became infected, usually later, presumably from contact with other sibs or persons outside the home whom they encountered more frequently with increasing age.

Among the HBsAg(+) mothers, there were apparent differences in infectivity for their babies in the following order: (1) HBsAg(+), (2) neither HBsAg(+) nor anti-HBs(+) and (3) anti-HBs(+) and (4) anti-HBe(−). This is consistent with studies in other parts of the world. The role of HBsAg in predicting infectivity appears to be independent of its lower frequency in this population. However, the frequency of transmission of HBV from HBsAg(+) mothers to their offspring is less in Africa than in Asia. In Asia, about 90% of babies born to HBsAg(+) women become HBsAg(+) by 3 months of age.

None of the infants had serologic markers of HBV infection before 5 months of age. This is somewhat different from babies in western populations and particularly from babies in Asian populations. In Taiwan, for example, more than 90% of babies born to HBsAg(+) mothers became HBsAg(+) by 3 months of age.

These results can now be viewed from the standpoint of recommending strategies for mounting a prevention program including the use of the hepatitis B vaccine. The offspring of carrier mothers become infected earlier than other children, but the offspring of other mothers also have a high likelihood of infection later in life. Mothers with anti-HB, will transmit the antibody to their children, particularly if it is in high titer in the mothers. However, anti-HB, appears to offer only a minimal amount of protection, and for only a few months. An observation of great importance is that none of the children we observed became infected before about 5 months of age. Therefore, vaccination before that age is advisable; it is known that children can muster an effective response to the vaccine during the first few months of life. Studies initiated by the late Professor Maupas and his colleagues indicated that such vaccinations prevented almost all HBV infections in the first 2 years of life. It appears that in Africa it will not be necessary to screen mothers to detect antigen or anti-HB, because all children in high-risk areas should be vaccinated regardless of the response of their mothers, and this vaccination should be done between 1 and 3 months of age. Furthermore, because infection does not occur until ≥5 months after birth, the use of hepatitis B immune globulin in babies born to HBsAg(+) mothers is not warranted.

Before any decision on the use of this vaccine is considered, it is necessary to evaluate the possible benefits against risks and costs of vaccination. The risk appears to be small. There is now experience with more than 500,000 vaccinations, and there do not appear to be any side effects of consequence. In West Africa, although hepatitis infection and its sequelae are important medical problems, malaria, schistosomiasis, infectious diarrheas, onchocerciasis, malnutrition, and other diseases may have higher priorities for public health resources. However, although hepatitis may not be the most important disease problem in West Africa, it may be one of the most important solvable disease problems. Population-based information of the kind provided by our study should be useful for planning the detailed tactics and strategy of prevention programs.

We thank Dr. Soumah, Mr. Souleymane Sy, Mr. Mamadou Sene, Dr. Bathilly, and Dr. Mustapha Sow for help and support.

REFERENCES
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ABSTRACT  An aqueous extract of the plant *Phyllanthus niruri* inhibits endogenous DNA polymerase of hepatitis B virus and binds to the surface antigen of hepatitis B virus in *vitro*. The extract also inhibits woodchuck hepatitis virus (WHV) DNA polymerase and binds to the surface antigen of WHV in *vitro*. The extract, nontoxic to mice, was tested for antiviral activity in woodchucks (*Marmota monax*). In a trial using six long-term WHV-carrier woodchucks, five treated animals showed a faster decrease in woodchuck hepatitis virus surface antigen titer compared to one untreated control. In animals recently infected with WHV, the extract was effective when administered i.p. in three out of four animals in reducing and within 3–6 weeks eliminating both the surface antigen titer and DNA polymerase activity in serum. The treatment was discontinued after 10 weeks, and the treated animals have remained free of detectable markers of WHV for more than 45 weeks. In contrast, three untreated controls remained positive for both markers for WHV. One of the controls died after 8 weeks; the other two controls have remained positive for WHV markers for more than 45 weeks. In a third trial with long-term carriers, test animals treated subcutaneously with the extract for 12 weeks did not respond; but on switching the mode of administration to i.p., two out of the five animals showed a significant decrease in woodchuck hepatitis virus surface antigen titer compared to controls.

Chronic carriers of the hepatitis B virus (HBV) may remain asymptomatic for long periods, but many are at high risk of eventually developing post-hepatitic cirrhosis and primary hepatocellular carcinoma. Carriers are often infected within the first few years of life, but symptoms of chronic liver disease and primary hepatocellular carcinoma may not be perceived until the third, fourth, or later decades; pathogenesis, even though relentless, is slow (1, 2).

Materials of animal, bacterial, and plant origin (3) have been described that appeared to interfere with the binding of the HBV surface antigen (HBsAg) to the HBsAg antibody (anti-HBs). Subsequently, about 1200 species of plant were tested, and about one-third were found to inhibit anti-HBs–HBsAg binding. To obtain more specificity and increase the probability of obtaining an effective therapeutic agent, in addition to the inhibition of HBsAg-anti-HBs binding, we examined plant extracts *in vitro* to determine if they inhibited the endogenous DNA polymerase (DNAp) of HBV, which is necessary for its replication. The first plant tested was *Phyllanthus niruri*, which has been and is used widely (4) in southern India and elsewhere for the treatment of jaundice. The treatment of HBV carriers has not been recognized in traditional indigenous medical systems. The inhibition of anti-HBs–HBsAg binding by *P. niruri* in *vitro* has been reported by Thyagarajan et al. (5).
Table 1. Effect of \textit{P. niruri} extract on the binding of HBs to HBsAg or WHsAg

<table>
<thead>
<tr>
<th>\textbf{P. niruri extract, mg/ml}</th>
<th>\textbf{% inhibition of anti-HBs binding}</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>61</td>
</tr>
<tr>
<td>2.5</td>
<td>45</td>
</tr>
<tr>
<td>1.25</td>
<td>25</td>
</tr>
<tr>
<td>0.63</td>
<td>11</td>
</tr>
<tr>
<td>0.5</td>
<td>13</td>
</tr>
<tr>
<td>0.31</td>
<td>13</td>
</tr>
<tr>
<td>0.1</td>
<td>5</td>
</tr>
</tbody>
</table>

Serum (50 \(\mu\)l) containing HBV (or WHV) particles was layered over a 10–20% sucrose gradient and centrifuged at 45,000 rpm for 3 hr in a SW 55 Ti rotor in a Beckman ultracentrifuge. Pelleted virus was separated from the supernatant and was resuspended in 5 \(\mu\)l of 0.05 M Tris-HCl, pH 8.0. Equal volume of serial dilutions of the extract of \textit{P. niruri} was mixed with the virus suspension. Then nucleotide triphosphates dATP, dGTP, dCTP, and \([^{32}\text{P}]\text{dTTP}\) were added in the presence of 0.05 M Tris-HCl, pH 8.0, containing 10 \(\mu\)M MgCl\(_2\), 0.15 M NaCl, 1 mM dithiothreitol, and 0.1% Nonidet P-40, and the mixture was incubated at 37°C for 2 hr. For control, the virus suspension was mixed with 0.05 M Tris-HCl buffer, pH 8.0, instead of the potential inhibitor. The reaction was stopped by the addition of 15 \(\mu\)l of Pronase (0.5 mg/ml) in 0.1% NaDodSO\(_4\) containing 0.01 M EDTA in Tris-HCl, pH 7.4; and the mixture was electrophoresed on 1.5% agarose gel using bromophenol blue as the tracking dye. The DNA formed was detected by autoradiography.

A quantitative determination of the inhibition was obtained by substituting \([^{3}H]\text{dTTP}\) and \([^{3}H]\text{dGTP}\) for \([^{32}\text{P}]\text{dTTP}\) as radiolabel according to the method reported by Hantz \textit{et al.} (10) with modifications. In this experiment the reaction was terminated by the addition of 15 \(\mu\)l of Pronase (0.5 mg/ml) in 0.1% NaDodSO\(_4\) containing 0.01 M EDTA in Tris-HCl, pH 7.4; and the mixture was electrophoresed on 1.5% agarose gel using bromophenol blue as the tracking dye. The DNA formed was detected by autoradiography.

To determine the WHV DNAp activity in sera of woodchucks used for experiments in vivo, a virus pellet, centrifuged from 50 \(\mu\)l of serum on sucrose density gradient, was used directly without addition of any diluent buffer as described above.

**RESULTS**

The Effect of \textit{P. niruri} Extract on WHsAg and HBsAg in Vitro. \textit{P. niruri} extract inhibits the reaction of HBsAg with anti-HBs and of WHsAg with anti-HBs (Table 1). The inhibition was concentration dependent for HBsAg and WHsAg.

Inhibition of WHV DNAp by the Extract in Vitro. Serial dilutions were assayed for their ability to inhibit WHV DNAp activity. Inhibition (Fig. 1) was directly proportional to the concentration of the extract up to 600 \(\mu\)g/ml at which the inhibition was 82%.

Toxicity Tests in Mice. Forty outbred albino female mice were used to determine the toxicity of the extract in accordance with a National Institutes of Health recommended assay to determine acute toxicity. The mice were divided into eight groups A and B. The numbers of the animals are given next to the appropriate curve.

![Fig. 1](image1.png)  
**Fig. 1.** Inhibition of DNAp of WHV by aqueous extracts of \textit{P. niruri}. The data are presented as \(\mu\)g (dry weight) per ml of assay mixture.

![Fig. 2](image2.png)  
**Fig. 2.** Effect of aqueous extracts of \textit{P. niruri} given i.p. on long-term chronic-carrier woodchucks (solid lines) compared to a single control carrier (dashed lines). For clarity, the results have been arbitrarily separated in two groups, A and B. The numbers of the animals are given next to the appropriate curve.
sets of five mice and were weighed. Four sets designated "test" were given i.p. aqueous extract of P. niruri [0.1 ml, 1.8 mg (dry weight) per mouse], while the other four sets, designated "control," were given i.p. PBS, 0.1 ml per mouse. All sets were weighed after 3 and 7 days. There was no loss in weight among the treated or control mice after 3 days. There was a net gain in weight among all the mice after 7 days. One of the treated mice died on the 3rd day of internal injury unrelated to the test.

Long-Term Chronic-Carrier Woodchucks. An initial study was conducted on woodchuck carriers whose sera showed the presence of both the surface antigen and WHV DNAp activity at the time these animals were trapped. The length of time these animals were carriers of WHV was unknown, but it was probably more than several months. Of the six carrier woodchucks that were available, five animals, 128, 129, 131, 228, and 250, were treated; and one, 274, was used as control. Test animals were given 0.5 ml of the extract [9 mg (dry weight)] i.p. once a week, while the control animal was given the same volume of buffered saline i.p. once a week. The animals were bled periodically, and the titer of WHsAg was determined (Fig. 2). A comparison of the slope of the curves indicated that the extract appeared to reduce the titer of WHsAg in long-term chronic carriers.

Woodchucks Recently Infected with WHV. The second study was conducted on animals that were negative for WHV at the time of capture but became positive while in the enclosures in our woodchuck colony. By the time of the experiment, these animals had been infected for at least 1 month. The aqueous extract of P. niruri [0.5 ml, 9 mg (dry weight) per woodchuck] was administered i.p. twice a week to four woodchucks, 319, 339, 360, and 376; the other three, 370, 388, and 117, received 0.5 ml of PBS twice a week. They were followed by weekly bleeding and assay for WHsAg and WHV DNAp. Treatment was terminated after 72 days, but the weekly bleedings were continued for over 300 days. Liver biopsy was performed on day 80, except for control woodchuck 117, which was autopsied when it died on day 57.

In woodchuck 319, WHsAg started dropping soon after the start of treatment, becoming undetectable about 21 days later (Fig. 3). WHV DNAp activity stayed positive for about 1 week after the surface antigen titer became undetectable, but subsequently it also became undetectable. Although the treatment with extract was terminated after 72 days, there were no detectable levels of the surface antigen or DNAp activity.
activity in 319 up to 300 days after the start of treatment (i.e., 228 days after termination of treatment). The control animal 370, on the other hand, did not show a drop in either WHsAg titer or DNAp activity up to 300 days.

Treated animals 339 and 360 showed a similar drop in WHsAg between 21 and 35 days after the start of treatment, followed 7-14 days later by a drop in WHV DNAp; the markers stayed undetectable up to 300 days. Control animals 117 and 388, however, showed high levels of the WHsAg and DNAp during the same period. Autoradiography of the product of DNAp reaction in the serial bleedings of control woodchuck 370 and P. niruri extract-treated woodchuck 339 is given in Fig. 4. The presence of a band at 3.3 kilobases (arrow) indicated WHV DNAp activity up to 21 and 28 days. These became undetectable thereafter. DNAp of control animal 370 did not change.

Woodchuck 376A, one of the four treated animals, became bacteremic early in the experiment. Chloramphenicol was administered, but the animal succumbed to the infection. It did not respond to the extract.

The histopathology of the livers of control woodchuck 370 and treated animal 339 are shown in Fig. 5. Data on the liver biopsy performed 8 days after the termination of treatment are given in Table 2. (Liver biopsies before treatment were not available.) The three untreated controls, 370, 388, and 117, showed extensive portal infiltration and focal necrosis; all three were diagnosed as chronic active hepatitis. The livers of the treated animals 319, 339, and 360, on the other hand, showed marginal or negative portal infiltration and focal necrosis. The diagnosis of woodchuck 319 was early mild viral hepatitis, of 339 was early active hepatitis, and of 360 was minimal portal hepatitis.

**Subcutaneous Administration of the Extract in Long-Term Carrier Woodchucks.** Five of eight long-term WHV-carrier woodchucks (327, 437, 456, 488, and 492) were administered 0.5 ml [9 mg (dry weight)] of extract subcutaneously twice a week. The remaining three (318, 429, and 471) were given 0.5 ml of PBS subcutaneously twice a week. The animals were bled weekly, and the titer of WHsAg and WHV DNAp was monitored. During 3 months of treatment, there was no appreciable change in either marker in the treated or control animals.

We concluded that subcutaneous administration was ineffective. We hypothesized that either the active principle was not absorbed by this route, that antibodies were developed against the extract, or some other mechanism rendered it ineffective. After 90 days the mode of administration was changed to the intraperitoneal route that had apparently been successful in the previous studies. Two of the treated animals showed a drop in WHsAg about 60 days after switching to i.p. administration (Fig. 6). One control and two treated animals died due to bacteremic infections. None of the control animals showed any significant change in WHsAg.

**DISCUSSION**

London and Blumberg (1) have proposed a model to explain the observations on the relation between primary hepatocellular carcinoma and HBV. It postulates the existence of fully differentiated liver cells which, when infected, allow complete replication of the virus and integration of virus DNA may occur. (They are designated S cells; i.e., susceptible to replication.) Less-differentiated liver cells (common in the fetus and newborn but less so in the adult liver), when infected, do not allow replication. Although penetration of the virus and integration of virus DNA may happen. A periportal lesion, with an agglomeration of lymphocytes, plasmacytes, histiocytes, fibroblasts, and necrotic hepatocytes (n); proliferating biliary ductules (d) are conspicuous, which, along with fibrosis, indicate a progression to cirrhosis. (x300.) Overall, these effects are much less than in the untreated liver. (D) Small perportal granuloma; hepatocyte and cords are well preserved, apart from rare necrosis (n). (x75.)

## Table 2. Data on liver biopsies performed 8 days after termination of treatment in experiment involving woodchucks recently infected with WHV

<table>
<thead>
<tr>
<th>Control animal</th>
<th>Portal infiltrate</th>
<th>Focal necrosis</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>370</td>
<td>±</td>
<td>+</td>
<td>Chronic, active hepatitis</td>
</tr>
<tr>
<td>388</td>
<td>+</td>
<td>+</td>
<td>Chronic, active hepatitis</td>
</tr>
<tr>
<td>117</td>
<td>+</td>
<td>+</td>
<td>Chronic, active hepatitis</td>
</tr>
<tr>
<td>P. niruri extract-treated animal</td>
<td>±</td>
<td>±</td>
<td>Mild viral hepatitis</td>
</tr>
<tr>
<td>319</td>
<td>+</td>
<td>+</td>
<td>Active hepatitis</td>
</tr>
<tr>
<td>339</td>
<td>±</td>
<td>0</td>
<td>Minimal, portal hepatitis</td>
</tr>
<tr>
<td>360</td>
<td>±</td>
<td>0</td>
<td>Extensive</td>
</tr>
</tbody>
</table>

0, None; ±, Marginal. +, Minimal positive. ++, Positive. +++, Extensive.
The proliferating HBV in the differentiated cells leads to cell death, in part caused by the immune response of the host to its S cells altered by HBV. The less-differentiated R cells are not damaged by the virus, since the virus does not replicate. The R cells divide and multiply in response to the death of the S cells. With increased division of R cells, chromosomes are more liable to disruption, deletion, rearrangement, or mutation by the resident HBV (or, possibly, another agent). This could result in a favored clone that divides rapidly and is eventually perceived as a cancer.

The course of these events could be stopped if virus were eliminated from the carrier. If this is not possible, then decreasing the virus load or inhibiting its entry into liver cells could slow the death of liver cells so that perceptible disease would not be expected to occur until the carrier had lived out a life span. We have referred to this as "prevention by delay" (2).

In an attempt to achieve this goal, we looked for agents that would affect the virus or its entry into liver cells. The aqueous extract of $P. \text{niruri}$ inhibits HBV DNAp and WHV DNAp and interferes with the binding of anti-HBs to HBsAg and WHsAg apparently because of its ability to bind the surface antigen.

The extract of $P. \text{niruri}$ was tested in three independent in vivo experiments in woodchuck carriers. In the first, with long-term carriers, a significant drop in the titer of WHSAg was observed in the extract-treated animals compared to the control. In the second, using short-term carriers, the WHSAg and WHV DNAp in three of four treated animals became undetectable and stayed that way even after the treatment was terminated, while the levels of these markers stayed high in the controls. These results indicated a possible break in the carrier state directly attributable to the treatment with $P. \text{niruri}$ extract. The conclusion from the third experiment was that the extract was not effective when administered subcutaneously. However, on switching the mode of administration to i.p., two out of three extract-treated animals showed a drop in WHSAg titer. The advantages of intraperitoneal chemotherapy for liver ailments have been reported (11) and may be applicable to WHV and HBV infections since replication of both these viruses takes place in the liver. Although this experiment was faulted because of its post hoc design, the results were in the direction predicted from the two earlier experiments.

Our preliminary results indicate that there are one or more active materials in $P. \text{niruri}$ that inhibit the replication of WHV in vivo and decrease the pathological effects of WHV on woodchuck liver. By inference, the substance should affect HBV infection in humans similarly. Using a variety of techniques (including HPLC), we have identified fractions from $P. \text{niruri}$ containing the DNAp inhibitory activity and the surface antigen binding activity.

We are indebted to Philip Custer for the histopathological studies, Howard Blatt for the biopsies, and Terry Halbherr and Renee Grob for technical assistance. The research was supported in part by grants from the Kaiser Family Foundation, the Mary L. Smith Charitable Lead Trust, and the Commonwealth of Pennsylvania.

EFFECT OF PHYLANTHUS AMARUS* ON CHRONIC CARRIERS OF HEPATITIS B VIRUS

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T. THIRUNALASUNDARI1 P. S. VENKATESWARAN2
B. S. BLUMBERG2

Department of Microbiology, Post Graduate Institute of Basic Medical Sciences, University of Madras, India; and Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA

Summary In a preliminary study, carriers of hepatitis B virus were treated with a preparation of the plant *Phyllanthus amarus* for 30 days. 22 of 37 (59%) treated patients had lost hepatitis B surface antigen when tested 15–20 days after the end of the treatment compared with only 1 of 23 (4%) placebo-treated controls. Some subjects have been followed for up to 9 months. In no case has the surface antigen returned. Clinical observation revealed few or no toxic effects. The encouraging results of this preliminary study recommend continued evaluation of this plant and the active principles isolated from it.

Introduction

Plants of the genus *Phyllanthus* have been used widely by traditional medical practitioners for the treatment of jaundice and other diseases. Their use was described in Indian Ayurvedic literature more than 2000 years ago. *Phyllanthus* species are also used in China, the Philippines, Cuba, Nigeria, Guam, East and West Africa, the Caribbean, Central America, and South America.

Extracts of *Phyllanthus amarus* and *P. tenellus* inhibit the reaction between HBsAg (the surface antigen of the hepatitis B virus [HBV]) and the antibody to it (anti-HBs). HBV contains an endogenous DNA polymerase which is required for its replication. *Phyllanthus* extracts inhibit the endogenous DNA polymerase of HBV virus, woodchuck hepatitis virus, and duck hepatitis B virus (unpublished). The inhibition is quantitatively specific for the DNA polymerases of these HBV-like viruses; much larger quantities are required to inhibit the DNA polymerases of bacteria and mammals (unpublished).

Five studies of the effect of extracts of *Phyllanthus* species on woodchuck (*Marmota monax*) carriers of woodchuck hepatitis virus have been completed (and published). The extract-treated animals were much more likely than placebo-treated controls to lose the virus, and the virus titres fell in more of the extract-treated than placebo-treated animals.

Acute (1 month) and chronic (3 months) toxicity studies in mice have been reported. There was no clinical or histopathological evidence of toxicity. The traditional medical uses indicate that this plant is non-toxic, although, to our knowledge, there have been no previous systematic studies of toxicity in man or animals. Various methods of administration are described in the traditional systems. In most of these methods the whole plant, rather than individual components, is used. Our eventual aim is to identify, purify, and synthesise an active agent from *Phyllanthus* which can be used in the treatment of HBV infection. We are concentrating on the fraction of the extract that inhibits the HBV DNA polymerase.

It is important to establish first whether the whole plant is effective in abolishing the carrier state. It is also possible that there are several active substances, or that several activities must act in conjunction for effective therapy or to prevent toxicity. Studies with the whole plant would contribute to an understanding of these questions.

*Phyllanthus* is used widely in India and elsewhere but, to our knowledge, there has been no controlled trial of its effectiveness in carriers. In this study, we have tested the hypothesis (stated and implied previously) that treatment with *P. amarus* will lead to the loss of the HBV carrier state.

Additional explanatory variables, particularly HBV serological responses, have also been measured to differentiate between responders and non-responders to the treatment.

Subjects and Methods

Carriers of HBV in three categories were identified. These included 28 symptom-free carriers detected among voluntary blood donors at the Institute of Child Health and the Hospital for Children, Madras; 14 carriers with symptoms, who were outpatients with the diagnosis of chronic liver disease at the Department of Gastroenterology, Government General Hospital, Madras; and 18 patients from the Institute of Child Health and Hospital for Children with glomerular nephritis, who were also chronic carriers of HBV.

Before the trial, the carriers were followed for 6 months with repeated (4–6) serological tests for HBsAg and anti-HBc IgM. Only those who remained carriers for 6 months were included in the study. The glomerular nephritis patients were followed for 1 year before inclusion. Carriers were stratified by sex and age (within 3 years) and assigned randomly to treatment and placebo groups. Treatment and follow-up were carried out with the subjects as outpatients. The treated patients were given 200 mg of the dried, powdered, sterilised plant in a capsule three times a day for 30 days. Placebo-treated patients were given an equal amount of sterile lactose in a capsule. The subjects were instructed to continue their
regular diet and activities. Of the 78 cases (40 treated, 38 placebo) randomised, 18 (3 treated, 15 placebo) failed to complete the course of capsules or did not return after the treatment. The population available for follow-up therefore consisted of 37 treated and 23 placebo subjects. All the clinical studies were done at Madras with plants collected locally.

Full-grown plants (30-45 cm) were selected. The whole plants, including roots, were quickly washed with cold water. A technique of intermittent sterilisation was used. The washed plants were maintained at 45°C for 30 min daily for 3 successive days. The roots were removed and the remainder of the plant powdered in an electric blender. The powder was dispensed under sterile conditions in 200 mg doses into gelatin capsules which had been presterilised with ethylene oxide. Samples of the dried powder were tested for aerobic and anaerobic bacteria and fungi by incubation in the appropriate media (blood agar plate, thioglycollate broth, Sabouraud's glucose agar). Randomly selected capsules from four batches of capsules were tested similarly on a regular basis. There was no evidence of contamination in any of the plant substance used for treatment. One batch of powder showed five colonies of Bacillus subtilis, and this batch was not used. Lactose was used as a placebo.

It was presented in gelatin capsules identical to those used for the plant powder in the same doses for the same time. Ninety capsules of plant or placebo were placed in a coded container. The doctors and nurses administering the substances were told to give a coded container from one or the other group alternately to carriers as they were admitted to the study. They were unaware who was receiving which treatment. The code was maintained at the Department of Microbiology which is 10-15 km from the hospitals where the capsules were given. The initial serological tests were carried out before the subjects were randomised. All the serological tests were done at the Department of Microbiology.

60 subjects returned for the first follow-up visit 15-20 days after the end of treatment. They were then asked to return each month. All 60 returned for the first 2 months. All but 1 treated and 4 placebo subjects returned for the third monthly visit and follow-up continued for some subjects for up to 9 months. At the first visit after treatment, each subject was examined clinically and a clinical questionnaire was completed with the help of the examining physician. The questionnaire was designed to determine whether any symptoms or signs occurred during treatment, including those that could be ascribed to side-effects or toxicity of the plant preparation. The information obtained included age, sex, and a history of jaundice, blood transfusions, surgery, and injections. A general medical history was also obtained. Symptoms which might be attributed to side-effects of the treatment included: fatigue, malaise, fever, chills, urticaria, anorexia, nausea, abdominal pain, diarrhoea, lymphadenitis, myalgia, arthralgia, headache, dizziness, disturbed sleep, paraesthesia, respiratory illness, and skin rash.

On each subsequent visit, the subjects were asked if they had had any symptoms or findings which might be attributable to the treatment. Blood samples were collected by venepuncture before treatment and at each follow-up visit.

Laboratory Methods

The serum was tested in Madras for HBeAg by enzyme-linked immunosorbent assay (ELISA; Auszyme H). Hepatitis B core antigen (HBeAg) by ELISA (HBe EIA), and IgM antibody against the core
antigens (anti-HBc IgM) by 'Corzyme-M' (Abbott Laboratories, North Chicago, Illinois, USA).

To exclude the possibility of systematic error in the determinations of HBsAg, the major outcome variable in this study, the serum samples that remained after the initial testing were also tested for HBsAg in Philadelphia. The western blot technique of McMichael et al., which requires only 3 μl serum, was used because only small amounts of serum remained for the confirmatory test. 42 samples were tested by this method. There was a very high correlation between the ELISA and western blot methods; all but 7 results were concordant. Of these, 4 showed a “trace” or “1 + ” score in the western blot compared with zero in the ELISA. The results of the ELISA method were used in the final analysis. To determine whether there were any differences in clinical chemistry between the treated and placebo groups, a series of studies were done on the blood in the pretreatment collection. Bilirubin, serum alanine and aspartate aminotransferases, and alkaline phosphatase were measured for the symptomatic carrier group and the glomerular nephritis group only. These included blood urea, serum creatinine, serum cholesterol, serum total protein, albumin, and globulin; 24 h urine protein, and blood glucose.

The results of the studies on the placebo and treated groups were compared by the Mann-Whitney statistic. The only significant difference was a higher level of bilirubin in the plant-treated group. From this we infer that there were no significant clinical differences between the groups.

Results

The demographic and serological characteristics of the subjects lost to follow-up and those who where followed for at least 2 months after the end of treatment are shown in table I. There were no significant differences between the groups. There was a significant difference between the placebo and treatment groups in the numbers defaulting (15 vs 3), but we are unable to explain why this occurred. According to the study design, the physicians and subjects were unaware of who was receiving the treatment or placebo, and there is no reason to believe that this rule was disregarded.

Overall, 22 (59%) of the 37 treated subjects compared with only 1 (4%) of the 23 placebo-treated patients had lost HBsAg at the first follow-up visit, a highly significant difference ($p < 0.0001$, Fisher’s exact test).

All subjects in the treated group, except for 1 symptom-free carrier, were followed for at least 3 months. HBsAg did not return in any of those who had lost HBsAg at the first follow-up visit. Follow-up has continued for up to 9 months in some subjects (table II), and there have been no cases of antigen reappearing.

In the placebo group only 1 of those followed up (a symptom-free carrier) had lost the antigen by the first follow-up visit, and she remained negative at the 3rd month follow-up (her last visit).

<table>
<thead>
<tr>
<th>Follow-up (mo)</th>
<th>Treated</th>
<th>Placebo</th>
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<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Overall, males and females appeared to respond equally, but in the symptomatic carrier group there was a suggestion of a poorer response by males. The numbers are too small to allow conclusions to be drawn on possible sex differences in response to treatment.

13 of the 14 plant-treated carriers (93%) who were positive for HBsAg but negative for HBeAg and anti-HBc IgM cleared the carrier state. Of the 9 treated carriers who were positive for HBsAg and HBeAg but negative for anti-HBc IgM, only 4 of 9 (44%) were cleared. Of the 8 who were positive for all three markers, only 1 (13%) was cleared. 4 of the 6 (67%) treated carriers positive for HBsAg and anti-HBc IgM but negative for HBeAg lost the carrier state. Overall, HBsAg-positive carriers with HBeAg were less likely to respond to treatment than those without HBeAg (5 of 17 [29%] vs 17 of 20 [85%]; $p < 0.001$). The single placebo-treated carrier who lost HBsAg had been positive for HBsAg only and was symptom-free.

The toxicity questionnaire described above was used during the follow-up visits. None of the treated or placebo groups seen at the first or subsequent follow-up visits had symptoms or signs attributable to toxic side-effects of the plant.

A rash developed in 1 treated subject, a boy aged 2-5 years, after 10 days of treatment. The treatment was discontinued. The child was found to be infected with roundworm, and his physician believed that the rash was a consequence of the infestation and not the Phyllanthus treatment. He was not included in the analysis.

Discussion

Our hypothesis has been supported by this preliminary study of human HBV carriers. Treated carriers were much more likely to lose the carrier state (as determined by the HBsAg ELISA) than those given placebo (59% vs 4%). Those that lost HBsAg remained clear for several months after the end of treatment. Follow-up on the carriers is continuing. Carriers with HBeAg were less likely to respond. The presence of anti-HBc IgM also appeared to reduce further the probability of clearance. Anti-HBc IgM
is thought to indicate recent infection, and presumably more active replication is taking place during this period. A smaller percentage of the carriers with evidence of viral replication (HBeAg) responded to treatment. This trial does not provide sufficient information on mode of action to explain this observation. It may be due to an inadequate dose and this can be tested in subsequent trials. Only 1 placebo subject cleared the virus.

We should consider the limitations of this preliminary clinical study. For reasons that are unclear, a significantly greater number of placebo subjects were lost to follow-up. On the basis of the experience with followed placebo subjects we would expect to see 1 subject among the placebo group not followed lose HBsAg. Although the inconsistent follow-up represents a potential flaw in the study, it seems unlikely that it could account for the highly significant difference between the treated and placebo groups.

The clinical and laboratory data collected in this preliminary study were limited by the facilities available in the outpatient services. However, the main outcome variables (HBsAg, anti-HBc IgM, HBeAg) were tested by sensitive and standard methods. It was not feasible to carry out extensive laboratory studies which might have detected side-effects of the medication. However, clinically, there were no obvious detrimental effects. This observation is supported by the lack of evidence of toxicity in the animal studies.13

These results must be regarded as tentative because of the flaws in design and execution already discussed, and the inherent uncertainty of trials of plant preparations, for example, possible inconsistency of dosage. They will, however, allow for a more rigorous design of future studies in India and elsewhere of the whole plant, and serve as a benchmark for eventual clinical trials of isolated and characterised active agents.

We thank Dr N. Madanagopalan (Department of Digestive Health and Diseases, Government Peripheral Hospital, Annanagar, Madras), Dr V. Ramalingam (Blotxl Bank), and Dr B. R. Nammalwar (Department of Nephrology, Institute of Child Health and Hospital for Children) for their collaboration with the clinical studies.

The work at Madras was supported by ICMR grants in IRIS nos: 8.301410. The work in Philadelphia was partly supported by USPHS grant CA-06Q27 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania.

Correspondence should be addressed to S. P. T., Department of Microbiology, Postgraduate Institute of Basic Medical Sciences, Taramani, Madras 600 113, India.

REFERENCES

EPIDEMIOLOGY AND POLYMORPHISMS
FILARIASIS IN MOENGO (SURINAM) IN 1950

by

B. BLUMBERG, J. McGIFF & I. GUICHERIT*

From the Medical Department of the Surinam Bauxite Company and Columbia University School of Public Health, New York City

(Received for publication July 25th, 1951)

Introduction

This paper is a report on the microfilaria survey conducted among the employees of the Surinam Bauxite Company and their families of Moengo, Surinam, during August and September, 1950. It reports on the distribution of microfilaraemia with respect to age, sex, race and geographical location with a discussion of the significance of these factors.

Materials and methods

Employees working night shifts were instructed to return when they were working days, in accordance with the observation that microfilariae appear in the night blood of day workers and in the day blood of those working nights. 1,019 smears were examined; 952 of a total of 971 employees listed on the payroll were examined. Of the remainder, six were reported as discharged, making an attendance response of 98.6 per cent. In addition, 67 staff members and their families residing in Moengo were examined.

The families of the employees, government officials and employees of the store, totaling 953 persons were also examined. The total population of Moengo, as of 31 December 1949, was 1,964. The total number of people included in this survey, resident in Moengo, was 1,716, or 87 per cent of the population.

The technique employed consisted of withdrawing 30 cu.mm. of finger blood between 10 and 11 p.m., making a thick drop smear, and staining with Giemsa stain. The entire blood smear was examined with the low power objective; reports were prepared of positive and negative smears, but no microfilaria counts were made.

Results

Of the total of 1,966 smears examined, 272 or 13.7 per cent were found positive for the microfilaria of Wuchereria bancrofti. In the employee group, 145 of 1,013 examined, or 14 per cent were positive; and in the family and resident group, 127 of 953, or 13.3 per cent were positive (see Table I). This slightly lower figure for the latter survey is probably due to the inclusion of younger age groups in which the rate of microfilaraemia is lower.

| TABLE I |
| Incidence of Wuchereria bancrofti in Moengo (Surinam, S.A) |
| Number examined | Employees (Survey I) | Residents (Survey II) | Total |
| Number positive | 1,013 | 953 | 1,966 |
| 145(14%) | 127(13.3%) | 272(13.7%) |

* With the technical aid of Mr. Yates.
The distribution by age of the microfilaria infection of the Moengo group is shown in the graph. The population is composed mainly of young men of working age, their wives and young children.

Table II shows the incidence of microfilaraemia by age groups. There is a marked rise in the 6 to 15 age group and a continuous relatively uniform infection rate throughout adult life.
TABLE III

W. bancrofti infection rates among males and females of all ages, Moengo, Surinam, S.A.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Number examined</th>
<th>Number positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>1,236</td>
<td>165 = 13.4%</td>
</tr>
<tr>
<td>Females</td>
<td>730</td>
<td>107 = 14.7%</td>
</tr>
</tbody>
</table>

Combining the survey results (Table III) the incidence for the males and females is found to be approximately the same, 13.4 and 14.7 per cent, respectively.

The age distribution is approximately the same for males and females.

TABLE IV

Distribution of W. bancrofti by race in employees and residents of Moengo

<table>
<thead>
<tr>
<th>Race</th>
<th>Employees</th>
<th>Residents of Moengo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total number</td>
<td>number of positives</td>
</tr>
<tr>
<td>Creole</td>
<td>606</td>
<td>121 = 20%</td>
</tr>
<tr>
<td>Indonesian</td>
<td>310</td>
<td>14 = 4.5%</td>
</tr>
<tr>
<td>Hindu</td>
<td>34</td>
<td>2 = 6%</td>
</tr>
<tr>
<td>Chinese</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>White</td>
<td>29</td>
<td>2 = 7%</td>
</tr>
<tr>
<td>American Indian</td>
<td>28</td>
<td>6 = 21%</td>
</tr>
<tr>
<td>Total</td>
<td>1,013</td>
<td>145 = 14%</td>
</tr>
</tbody>
</table>

The racial distribution of the groups covered in the survey is shown in Table IV. Creoles and Indonesians form the predominant groups while Hindus from India, Chinese, aboriginal Indians and whites are present in much smaller numbers. This survey indicates a much higher infection rate in the Creoles than in any of the other groups, the second highest rate being in the aboriginal Indians.

TABLE V

Comparison of the infection rate among Creoles and Indonesians, Moengo, Surinam

<table>
<thead>
<tr>
<th>Race</th>
<th>Number examined</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creole</td>
<td>1,278</td>
<td>237 = 18.5%</td>
</tr>
<tr>
<td>Indonesian</td>
<td>249</td>
<td>9 = 3.6%</td>
</tr>
</tbody>
</table>

Table V gives the data on the two major groups, Indonesian and Creole, living in Moengo under the similar environmental, social, and economic conditions which obtain in the company built and maintained concession. The age distribution of the two groups is approximately the same. The Creole infection rate was 18.5 per cent and that of the Indonesians 3.6 per cent. The percentage of microfilaraemia among the Creoles is 5.1 times as great as among the Indonesians. This marked difference in the incidence of microfilaraemia between these two racial groups living under apparently similar environmental conditions has not to our knowledge been commented on previously in the literature.
The results obtained in the survey of employees provides a comparison of the group residing in Moengo with those of the surrounding locations (see Table VI).

<table>
<thead>
<tr>
<th>Residence area</th>
<th>Number examined</th>
<th>Positives</th>
<th>Racial group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moengo</td>
<td>639</td>
<td>115 (= 18%)</td>
<td>Creole</td>
</tr>
<tr>
<td>Julianadorp</td>
<td>44</td>
<td>12 (= 27%)</td>
<td>Creole</td>
</tr>
<tr>
<td>Moengo and Julianadorp</td>
<td>683</td>
<td>127 (= 18.5%)</td>
<td>Creole</td>
</tr>
<tr>
<td>Overkant</td>
<td>20</td>
<td>1 (= 5%)</td>
<td></td>
</tr>
<tr>
<td>Staff Village</td>
<td>63</td>
<td>3 (= 4.7%)</td>
<td>White and Creole</td>
</tr>
<tr>
<td>Wonoredjo (Indonesian)</td>
<td>235</td>
<td>14 (= 5%)</td>
<td>Indonesian</td>
</tr>
<tr>
<td>Farm</td>
<td>12</td>
<td>0</td>
<td>Hindu</td>
</tr>
</tbody>
</table>

Moengo includes all the company and government houses in Moengo proper, exclusive of Julianadorp, Staff Village, and Farm. These are frame houses with some screen protection, raised off the ground with adequate sanitary and washing facilities. Pipe-line water is from deep wells. Uncovered rainwater receptacles alongside some of these residences provide a source of mosquito breeding. Complete DDT spraying is conducted twice yearly.

Julianadorp consists of two-storey, two-family residences completed within the last year. They are of concrete block construction and the sanitary and other controls are the same as for Moengo. The residents of this section have but recently moved here from Moengo and for all purposes may be considered with this group.

Wonoredjo, or Lynweg, is the Indonesian settlement directly adjoining Moengo to the East. The dwellings are mainly thatch and frame with inadequate sanitary facilities and for the most part no running water. The inhabitants are mostly Indonesian with a few Creoles and Chinese. There are many mosquito breeding areas in the settlement. DDT spraying is conducted here also.

Overkant includes the dwellings on either side of the Cottica River above and below Moengo. These are thatch buildings with inadequate sanitary facilities, little mosquito protection and no running water. Mosquito breeding areas abound.

The Staff Village consists of well-built, well-screened homes of the local, European, and U.S.A. staff members, with excellent sanitation and mosquito protection and water facilities. There are few mosquito breeding areas.

The Farm consists of residences for the farm workers adjoining the cow barns and similar in construction and equipment to the Moengo dwellings. The inhabitants are mainly Hindus (originating from India).

The highest rates occur in Moengo and Julianadorp (18 and 27 per cent) where the Creoles are the predominant group, whereas the other areas have the lower rate of approximately 5 per cent. The incidence rates in these different localities appear to parallel the incidence rates peculiar to the predominant racial group in the residence area. Whether this represents a racial or environmental factor remains to be decided in further studies.
Summary and Conclusions

(1) There is a 13.7 per cent microfilaraemia rate in the employees of the Surinam Bauxite Company and their families.
(2) There is a higher microfilaraemia rate in the Creoles than in the Indonesians.
(3) There appears to be no difference in microfilaria incidence in males and females.
(4) The microfilaraemia incidence increases after the age of six.
(5) The microfilaraemia rates of the different geographical areas appears to parallel the rates for the predominant groups residing in that area.

Acknowledgements

The cooperation of Mr. I. R. J. de Greve, Managing Director of the Surinam Bauxite Company, Mr. van der Lely, Plant Manager, Moengo Works, and the Staff of the Surinam Bauxite Company is gratefully acknowledged.

Special thanks are due to Dr. E. Vas, physician at the Moengo Hospital; Mr. Jungerman, pharmacist; Sister Relyveld; Sister Fernandes; Sister Aerst; Attendant Dompig; Attendant van Hetten; Assistant Limes, and the rest of the nursing and auxiliary staff of the Moengo Hospital.
OPPORTUNITY arose during recent trips to Iceland and Nigeria to collect samples of milk from the indigenous herds of cattle. These were examined to determine the type or types of β-lactoglobulin they contained.

There are two β-lactoglobulins, namely, β_A and β_B, distinguishable by paper electrophoresis. Aschaffenburg and Drewry have studied their incidence in British breeds, and by family studies have demonstrated their genetic determination. Their presence or absence is determined by two allelic autosomal genes, and all three phenotypes, β_A, β_B and β_A.B, have been identified.

Samples of milk were prepared for paper electrophoresis according to the method of Aschaffenburg and Drewry, and run on Whatman 3_M_M paper for 17 hr. at 2 m.amp./cm. width of paper and a potential drop of about 1.5 V./cm. length of paper, in veronal buffer I = 0.05, pH 8.6 in a 'Durrum'-type apparatus. Staining was with alcoholic bromphenol blue, and washing with 2 per cent acetic acid and tap-water.

The Nigerian cattle were White Fulani or Lyre-horned Zebu from the stock farm of the Veterinary Research Station, Vom. They are big humped animals, with large dewlaps and frequently extremely large horns. They are similar in appearance to the Brahmin cattle of the East. They are thought to have been brought into Nigeria by the nomadic Fulani people. Twelve of the specimens were from White Fulani-Friesian crosses.

Samples from Icelandic cattle were collected from various dairy herds on the island. The Icelandic breed is thought to have originated in Norway, and to have been isolated for a considerable period. The Icelandic cattle were selected so as to be free from close interrelationships. Some sister and mother-daughter pairs were present in the Nigerian cows.

The distribution of the β-lactoglobulin phenotypes is shown in Table 1. They fit the predictions of the Hardy-Weinberg law quite well. In the Nigerian sample the Friesian-Fulani crosses are included. Their omission does not significantly alter the results.

By B. S. BLUMBERG* and M. P. TOMBS
Department of Biochemistry, Oxford

### Table 1. Incidence of β-LACTOglobulins and α-LACTOAlBumin in Nigeria (White Fulani) and Icelandic Cows

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Nigeria (W. Fulani)</th>
<th>Iceland</th>
<th>British breeds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Predicted incidence</td>
<td>Predicted incidence</td>
</tr>
<tr>
<td>β_A</td>
<td>3</td>
<td>2.4</td>
<td>0.941</td>
</tr>
<tr>
<td>β_A.B</td>
<td>18</td>
<td>18.4</td>
<td>0.321</td>
</tr>
<tr>
<td>β_B</td>
<td>37</td>
<td>37.0</td>
<td>0.638</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>58.4</td>
<td>0.941</td>
</tr>
</tbody>
</table>

P = 0.9

### Table 2. Incidence of the α-LACTOalbumin and β-LACTOglobulin Phenotype in Forty-six Pure Breed White Fulani (Zebu) Cows

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>α_A</th>
<th>α_A.B</th>
<th>α_B</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>β_A</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>β_A.B</td>
<td>1</td>
<td>0</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>β_B</td>
<td>0</td>
<td>12</td>
<td>19</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>12</td>
<td>33</td>
<td>46</td>
</tr>
</tbody>
</table>

* Fellow of the Arthritis and Rheumatism Foundation.
\( \alpha \)-lactalbumin. No fast \( \alpha \)-lactalbumins were found in the Fulani-Friesian crosses. The incidence of each of the phenotypes in the pure Fulani animals only is shown in Table 1.

The distribution of the three types agrees closely with the predictions of the Hardy-Weinberg law. Although too few samples have been examined to provide family studies, by analogy with the case for the \( \beta \)-lactoglobulins, it is suggested that these \( \alpha \)-lactalbumins are also genetically determined. The symbols \( \alpha_A, \alpha_B \) are given to the proteins, and the symbols \( L^A_A, L^B_B \) assigned to the genes presumably determining their presence. \( \alpha_A \) is the faster moving component and \( \alpha_B \) the slower on paper electrophoresis under the conditions stated above.

All twelve paired \( \alpha \)-lactalbumins were found in animals which produced only \( \beta \)-lactoglobulin. This association is significant at less than the 5 per cent level. The one sample with only \( \alpha_A \)-lactalbumin was found in a \( \beta_BA \)-producer (Table 2).

This might be genetic association through close linkage with selection or due to some physiological effect in the mammary gland. The fast-moving \( \alpha_A \)-lactalbumin has not so far been found in any British breed, nor in the Icelandic. This protein may therefore provide a marker by which the affinities of the Zebu cattle, and indirectly the Fulani people associated with them, may be traced. The \( \beta \)-lactoglobulins do not appear to be suitable for distinguishing the breeds so far examined. No selective value has yet been found for either the \( \alpha \)-lactalbumins or the \( \beta \)-lactoglobulins.

This investigation was supported in part by a research grant, H-3161, from the National Heart Institute, U.S. Public Health Service, and the Wenner-Gren Foundation for Anthropological Research. Milk samples were collected from the Nigerian cattle with the permission and help of Dr. J. Taylor, director of the Veterinary Research Station, Vom. One of us (M. P. T.) is indebted to the Agricultural Research Council for a travel grant, and to the Icelandic authorities and Mr. B. S. Benedikz for their assistance in Iceland. Investigations in Nigeria were carried out under the auspices of Prof. B. G. T. Elmes, Department of Pathology, University College, Ibadan.

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HAPTOGLOBIN TYPES IN BRITISH, SPANISH BASQUE AND NIGERIAN AFRICAN POPULATIONS

By Dr. A. C. ALLISON*, Dr. B. S. BLUMBERG† and Mrs. ap REES

Department of Biochemistry, University of Oxford

SMITHIES,† found that the patterns obtained by starch gel electrophoresis of sera from different human subjects fall into three groups. The determinant proteins combine with hemoglobin and appear to be identical with the haptoglobins previously studied by Jayle and his colleagues.† It has therefore been suggested by Smithies and Walker that the groups should be named haptoglobin types 1-1, 1-2 and 2-2 (formerly types I, IIa and IIb, respectively). Preliminary genetical analysis indicated that the groups correspond to the three genotypes produced by a single pair of allelic genes. In fact, our unpublished observations suggest that the genetical control of the haptoglobin types is more complex. A detailed discussion of the evidence will shortly be submitted for publication. In the meantime results can be expressed in the nomenclature of Smithies and Walker, with the addition of a fourth group of subjects in which no haptoglobin at all can be detected, for which the designation type O-O is suggested.

The frequency of the haptoglobin types in different populations is of anthropological and genetical interest. It may also provide a clue to the way in which this remarkable polymorphism is maintained. The only published population studies of which we are aware are those of Smithies on Canadians and of Sutton et al. on Americans and Africans from Liberia and the Ivory Coast. In this article we report on the results of haptoglobin type analyses of 218 British subjects, 107 Spanish Basques and 99 Nigerian Africans presented.

The British sera were taken from unselected laboratory staff and blood donors in the Oxford region. The Basque bloods were collected as described elsewhere. The African sera were taken from Yoruba of Ilobi, Western Nigeria.

Fresh oxyhemoglobin was added to all sera to give a final concentration of the order of 100-120 mgm. per 100 ml. before electrophoresis in starch gels. Each gel was then cut horizontally and one half stained with benzidine, as described by Allison and ap Rees. The addition of enough hemoglobin nearly to saturate the haptoglobins, but not much more nor less, and benzidine staining of the gels after electrophoresis, greatly facilitates the identification of the haptoglobin types, which may not otherwise be easy. As will be mentioned below, the discrepancy between our results and those of Sutton et al. on West Africans may be attributable to differences in technique.

The results of our three surveys, together with those of Smithies on Canadians and Sutton et al. on Americans and Africans from Liberia and the Ivory Coast, are summarized in Table 1. The frequencies of the various haptoglobin types in the British series are not significantly different from those in the small Canadian and American series, although the former includes six subjects of type O-O, none of which was described by Smithies or by Sutton et al. The frequencies in the Basque series also are not significantly different from those in the British, Canadian and American series. Thus the uniqueness of the

Table 1. HAPTOGLOBIN TYPES IN VARIOUS POPULATIONS

<table>
<thead>
<tr>
<th>Population</th>
<th>No. tested</th>
<th>0-0 (Per cent)</th>
<th>1-1 (Per cent)</th>
<th>1-2 (Per cent)</th>
<th>2-2 (Per cent)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canadian</td>
<td>49</td>
<td>0</td>
<td>21-1</td>
<td>50-5</td>
<td>14</td>
<td>Smithies</td>
</tr>
<tr>
<td>American</td>
<td>54</td>
<td>0</td>
<td>11-1</td>
<td>55-5</td>
<td>19</td>
<td>Sutton et al.</td>
</tr>
<tr>
<td>British</td>
<td>218</td>
<td>6</td>
<td>10-1</td>
<td>48</td>
<td>13</td>
<td>Allison et al.</td>
</tr>
<tr>
<td>Basque</td>
<td>107</td>
<td>6</td>
<td>121</td>
<td>44</td>
<td>42</td>
<td>Allison et al.</td>
</tr>
<tr>
<td>Liberian and Ivory</td>
<td>142</td>
<td>0</td>
<td>48-7</td>
<td>42-3</td>
<td>13</td>
<td>Sutton et al.</td>
</tr>
<tr>
<td>Coast (African)</td>
<td>99</td>
<td>32</td>
<td>53-5</td>
<td>11</td>
<td>3</td>
<td>Allison et al.</td>
</tr>
<tr>
<td>Nigerian (African)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The frequency of the haptoglobin types in different populations is of anthropological and genetical interest. It may also provide a clue to the way in which this remarkable polymorphism is maintained. The only published population studies of which we are aware are those of Smithies on Canadians and of Sutton et al. on Americans and on West Africans from Liberia and the Ivory Coast. In this article we report on the results of haptoglobin type analyses of 218 British subjects, 107 Spanish Basques and 99 Nigerian Africans presented.

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The results of our three surveys, together with those of Smithies on Canadians and Sutton et al. on Americans and Africans from Liberia and the Ivory Coast, are summarized in Table 1. The frequencies of the various haptoglobin types in the British series are not significantly different from those in the small Canadian and American series, although the former includes six subjects of type O-O, none of which was described by Smithies or by Sutton et al. The frequencies in the Basque series also are not significantly different from those in the British, Canadian and American series. Thus the uniqueness of the
in the urine would be common among Africans. To our knowledge, this point has not been investigated.

The fact that several haptoglobin types exist side by side in a number of different populations indicates that it is a true polymorphism. This may have been maintained, like those of the blood groups and abnormal hemoglobins, by a balance of selective forces related to susceptibility to disease. The absence of haptoglobins might be disadvantageous, particularly in regions where there is a deficiency of iron in the diet. The fact that so many Nigerians lack haptoglobins is presumably due to a compensatory advantage in persons heterozygous for the factor concerned. The low frequency of type 2-2 in the tropics suggests that it may confer protection against one or more conditions common in temperate, but not in tropical, climates.

Dr. F. Alberdi kindly co-operated in the collection of the Basque blood specimens and Drs. Jean Grant and H. Preston in the collection of British bloods. The Nigerian specimens were collected by one of us (B. S. B.) as part of the work carried out on an expedition supported by the Wenner Gren Foundation, Inc., and grant No. H-3161 from the National Heart Institute, U.S. Public Health Service. The help of Prof. B. G. T. Elmes, Ibadan, is gratefully acknowledged.

Distribution of Electrophoretically Different Haemoglobins among Some Cattle Breeds of Europe and Africa

Two haemoglobins, A and B, can be distinguished in the blood of cattle by means of paper electrophoresis. It has been shown that the presence or absence of the haemoglobins is determined by two allelic autosomal genes which are fully expressed in the heterozygote. The three phenotypes, bovine A (slow moving component in electrophoresis), bovine B (fast moving component in electrophoresis), and bovine AB (both haemoglobins present), have been found. In a previous study, it was shown that among British breeds, only Jerseys, Guernseys and South Devons possessed the B haemoglobin.

In Table 1, the distribution of haemoglobin genotypes in the polymorphic European and African cattle breeds is shown.

Table 1. The Distribution of Haemoglobin Genotypes in the Polymorphic European and African Cattle Breeds

<table>
<thead>
<tr>
<th>Location</th>
<th>Breed</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AA</td>
</tr>
<tr>
<td>Jersey</td>
<td>Jersey</td>
<td>32</td>
</tr>
<tr>
<td>N. France</td>
<td>Brittany Shorthorn</td>
<td>0</td>
</tr>
<tr>
<td>X. France</td>
<td>Flamande</td>
<td>8</td>
</tr>
<tr>
<td>Mid France</td>
<td>Limousine</td>
<td>13</td>
</tr>
<tr>
<td>Mid France</td>
<td>Charolais</td>
<td>9</td>
</tr>
<tr>
<td>Mid France</td>
<td>Parthenaise</td>
<td>10</td>
</tr>
<tr>
<td>S. France</td>
<td>Salers and Aubrac</td>
<td>10</td>
</tr>
<tr>
<td>S. France</td>
<td>Garronaise</td>
<td>2</td>
</tr>
<tr>
<td>S.E. France</td>
<td>Tarentaise</td>
<td>9</td>
</tr>
<tr>
<td>S.E. France</td>
<td>Brown Swiss</td>
<td>2</td>
</tr>
<tr>
<td>Switzerland</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>W. Africa</td>
<td>Zebu (White Fulani)</td>
<td>32</td>
</tr>
<tr>
<td>E. Africa</td>
<td>Sudan Zebu</td>
<td>13</td>
</tr>
</tbody>
</table>

* Low tolerance to trypanosomiasis infection.

some selective factors and especially factors favouring the heterozygote may be operating under natural conditions or under those of man-controlled breeding. No such selective factors have yet been confirmed, although in the present study a possible association with lack of tolerance to trypanosomiasis in some African breeds has been noted.

The haemoglobins were detected by a modification of the starch gel electrophoresis method of Smithies. A drop of blood, collected from the jugular vein or by ear puncture, was placed on a 2-in. circle of Whatman filter paper, No. 1 or 3, and, when dry, forwarded by air to the laboratory in England. On arrival, 5 mm. circles of the dried blood were punched out of the filter paper and inserted into the starting slit of the starch gel. Borate buffer pH 8.0, ionic strength 0.06, was used for the preparation of the gel and borate buffer pH 8.6 was used in the electrode bath solution. Readings were made 4 hr. later, and provided that a control sample of polymorphic haemoglobin was included in all runs, excellent identification of haemoglobin was possible on dried samples up to one month old (Fig. 1). Identifications using this method were checked against determinations on whole blood using paper electrophoresis and were found to be consistent.

In Table 1, it will be seen that bovine B haemoglobin is present in many European herds, but its frequency is low compared with that found in Jersey Island cattle. The high haemoglobin B frequency again appears in breeds of Zebu stock.

The possible presence of selective factors may affect the use of the haemoglobins as simple genetic markers of breed inter-relationships. However, the presence of the B gene in breeds located geographically on a
line running southwards through France, and again in breeds in West and East Africa, is consistent with at least one of Boston’s suggested genealogical lines of the Jersey breed. As it is probable that hemoglobins bovine A and B are similar to the two described by Cabannes and Serain, there is yet further evidence of the B gene in Africa, although in this case it may have been imported into Algeria by Europeans through the medium of French breeds. On the other hand, and in view of the apparent mode of inheritance of the hemoglobin genes, it is improbable that the cattle breeds of north-east France, Holland and Denmark have in recent times received any genes from the cattle of Jersey, central and southern France, or of cattle of Zebu stock (Table 2).

Although little is known of the nature or tolerance of cattle breeds to trypanosomal infection, there is evidence that N ‘Dama and Muturwa have a greater tolerance to the disease than do the White Fulani (Zebu) cattle. This receives further support from the quantitative studies of Desowitz, on the immune reactions of N ‘Dama and Zebu breeds to trypanosomal infections.

The present study shows a prima facie correlation between the absence of bovine B hemoglobin and relatively high tolerance to trypanosomiasis (see Tables 1 and 2) and assumes some interest when related to the work of Allison on the presence of sickle cell hemoglobin and resistance to falciparum malaria in man. In order to determine if this is more than coincidence, it will be necessary to study, first, other breeds in the same environment; second, the relationship between the hemoglobin phenotypes and tolerance to disease in members of the African breed; and third, breeds presumably related to the Zebu in areas remote from trypanosomiasis exposure. It has recently been shown that Nigerian White Fulani (Zebu) cattle are polymorphic for the genetically determined milk proteins \( \alpha \)-lactalbumin A and B, as are some European breeds, and that they are the only breed so far studied to have two types of \( \alpha \)-lactalbumin, which may also be genetically determined. A parallel study of the distribution of these proteins may help in the problems outlined.

It is with pleasure that we acknowledge the help given by Mr. N. Le Q. Blampied, Mr. C. D. Cooke, Dr. M. Theret, A. A. Karib, H. K. Littlewood, A. G. Wilder and Dr. Hans Fey. We would also like to thank those breeders and owners who so readily offered material for this survey; for the loyal and patient technical help of A. Bernard; and for the invaluable help of O. Oyefeso in the field work in Nigeria. Prof. B. G. T. Elmes, University College, Ibadan, was instrumental in arranging the collections in Nigeria. The investigations in Nigeria were supported in part by a research grant from the Wenner–Gren Foundation for Anthropological Research and by a Research Grant, H–16 of the National Heart Institute, U.S. Public Health Service. A grant was made to one of us (A. D. B.) by the Agricultural Research Council for visits to France.

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AN ISOPRECIPITATION REACTION DISTINGUISHING HUMAN SERUM-PROTEIN TYPES

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During the past decade it has become apparent that human beings differ in their serum-proteins as well as in their red blood-cells. Genetically controlled variations have been described in haptoglobins and transferrins (Smithies and Connell 1959), \( \gamma \)-globulins (Grubb 1957, Ropartz 1960), and in the Gc system definable by immunoelectrophoresis (Hirschfeld and Beckman 1960). Though the chemical differences between the alternative proteins are not yet known, they are generally assumed to be small—analogous to the single aminoacid substitutions that distinguish the different human haemoglobin half-molecules (Hunt and Ingram 1959) and the small sugar substitutions that distinguish the blood-group substances (Morgan 1959). Nevertheless, the possibility remains that these or other serum-proteins are sufficiently different in structure to induce serum-protein isoimmunisation when blood is transfused.

Such a reaction is already known in rabbits. Oudin (1956), Dray and Young (1959), and others have shown that rabbits can be immunised with serum from other rabbits of different \( \gamma \)-globulin type. In the original experiments the foreign plasma was introduced with adjuvants, but recent observations (S. Dray, personal communication) have shown that adjuvants are unnecessary for immunisation. Three or four intravenous injections of serum from rabbits of another \( \gamma \)-globulin type led to the production in recipients of well-defined precipitating antibodies.

The experiments described here were undertaken to find out whether any similar reaction can be shown in man. Sera were obtained from subjects who had received multiple transfusions and were tested for precipitating antibodies against a panel of sera chosen to represent all the available genetically controlled serum-protein types. A serum has been found which gives strong precipitation with some, but not all, sera; and this reaction defines a polymorphism involving the \( \alpha_2 \)-globulins. Besides being of genetic interest, this finding raises the possibility that serum-protein isoimmunisation takes place in man and represents a clinical problem in persons receiving blood-transfusions.

Materials and Methods

Post-transfusion sera.—These were obtained from patients at the Clinical Center of the National Institutes of Health, Bethesda, who had received 5 or more transfusions. The indications for transfusion included open-heart surgery, haemolytic and aplastic anaemias, pelvic tumours, and leukae- mia. Wherever possible, blood-samples were drawn between 10 and 30 days after the last transfusion. Sera were stored at −20°C and were tested within 5 days of collection. Many of the subjects had received 30 or more transfusions.

Panel sera.—These were selected from the collection of the Section on Geographic Medicine and Genetics of the National Institute of Arthritis and Metabolic Diseases. The 24 sera included haptoglobin types 1-1, 2-1, 2-2, 2-1M, and O; transferrin types CC, CD, and BC; and \( \gamma \)-globulin types Gm \( \text{a} + \), Gm \( \text{a} - \), Gm \( \text{b} + \), Gm \( \text{b} - \), Gm \( \text{x} + \), and Gm \( \text{x} - \). In addition, 3 sera from patients with definite rheumatoid arthritis having high titres of rheumatoid factor, and 1 serum from a patient with well-developed lupus erythematosus (l.e.) having positive l.e. histological preparations and high titres of antinuclear antibodies, were used. The sera had been stored at −20°C for periods varying from 2 days to 2 years. Storage did not alter the reaction to be described. The sera were from different population groups, including American whites (sixteen), American Negroes (three), Micronesians (three), an Alaskan Eskimo, and a Viet-Namese.

Gel-diffusion precipitation technique.—The Ouchterlony procedure was carried out in 5 cm. petri dishes containing 8 ml. of 0.9% Oxoid Ionagar (w/v) in 0.07 M sodium phosphate buffer, pH 7.0, with 0.001 M sodium ethylenediaminetetra-acetate and 0.001 M sodium azide. With a die, six wells 6 mm. in diameter were cut round a centre well of the same size. The circumferences of the centre well and peripheral wells were 4 mm. apart. After removal of the agar cores from the wells, the bases of the latter were sealed with small volumes of molten agar. The post-transfusion sera were placed in the centre wells and panel sera in the peripheral wells. The plates were stored at room temperature and observed at 18 hours (by which time clear-cut precipitation was usually visible), 48 hours, and 4 days. The precipitation is best seen by oblique illumination from below against a dark background.
Immunoelectrophoresis.—This was done by the micromethod of Grabar and Williams (1955) and Scheidegger (1955) using standard lantern-slide plates coated with agar made up in barbitone buffer 0.01 M, pH 8.4. Antibody (or antigen) was placed in the side well and examined after reacting for 18 hours, by which time well-defined precipitates were visible. The reaction was continued to 36 hours, after which the plates were washed in saline and stained.

Starch-gel electrophoresis.—Starch gel (Connaught Laboratories, Toronto) was made up in tris-(hydroxymethyl)-amino-methane buffer (Poulik 1957) and poured into 60 x 80 x 150 mm. trays. Samples were inserted as a starch paste (Smithies 1955) into a slot 3 mm. wide. Electrophoresis was carried out for 5 hours at 6 V per cm. The position of the components was identified in a small strip of gel; the various components were eluted by the method of Gordon (1960) and were concentrated by ultrafiltration.

Preparation of γ-globulin components.—Separation of 7S and 19S γ-globulins was achieved by sucrose density-gradient centrifugation (Fudenberg and Kunkel 1957) using 40, 30, 20, and 10%, solutions of sucrose.

Bentonite-flocculation test.—The method of Bozicevich et al. (1958) was used.

Latex-fixation test.—This was carried out as described by Singer and Plotz (1956), using the Hyland 'RA-test' kit (Hyland Laboratories, Los Angeles, California).

Observations

A precipitin was found in the serum of a patient who had received many transfusions. He had a long and varied history of illness. The patient is a 64-year-old white American retired executive of Hungarian birth. At the age of 16 he had albuminuria which lasted for 4 years. In 1934, while still living in Budapest, he had an episode of sudden onset of paresthesia and paralysis of the legs. This resolved spontaneously in 2 months. A second episode, involving complete paralysis up to the waist, weakness of the arms, and loss of sensation and reflexes in the legs, took place in the United States in 1958. Cerebrospinal fluid contained 73 mg. protein and 150 lymphocytes per ml. Polynu-eritis of unknown cause was diagnosed, and again remission was spontaneous.

The patient also had symptoms of peptic ulceration dating from 1921, with gastrointestinal hemorrhage in 1938 and 1940. In 1941 a gastroenterostomy was performed, but symptoms and hemorrhage have recurred at intervals. The patient was again admitted to hospital in 1958, at which time he was reported to have low red-cell counts, low hemoglobin levels, and high white-cell and platelet counts. X-ray examination suggested an ulcer crater in the proximal duodenum. The bleeding continued for a month and then ceased, and with the help of blood-transfusions the hemoglobin was raised to 14 g. per 100 ml. Because of the high white-cell count and the presence of numerous megakaryocytes in the bone-marrow and some in the peripheral blood, the possibility that the patient might have leukaemia was raised. No convincing evidence that this is so has been obtained.

Without transfusion the patient's hemoglobin continued to fall, and in February, 1958, he was admitted to the Clinical Center of the National Institutes of Health. During his time in hospital the patient had persistent anemia without reticulocytosis. The leucocyte count varied from 10,000 to 15,000 and the platelet count was consistently above 500,000 per c. mm. The differential count showed predominantly neutrophils with a relative increase of immature forms. Bone X-rays showed no abnormalities. Refractory anemia of unknown cause was diagnosed. The patient was given cells from 4 units of blood, which raised his hemoglobin to 11 g. per 100 ml., and was discharged.

When the patient first received blood-transfusions in 1958 they were well tolerated. Since that time he has received 47 units of blood. The patient is of blood group AB, Rh +, and there has never been evidence of red-cell incompatibility with donor bloods. In the spring of 1960 the patient began to have transfusion reactions. Within about 1 hour of some, but not all, transfusions there was a fever, rising to 38-39°C (100-4-102°F) accompanied by headache and muscle pain, but no other symptoms. The fever and symptoms persisted for about 5 hours, and then slowly subsided. The patient had been discharged from hospital before the precipitin in his blood was discovered, so that it has not yet been possible to ascertain whether only bloods containing the precipitating antigen give transfusion reactions.
Precipitation Reactions

The serum of the patient gave well-defined precipitation reactions in agar with some, but not all, panel sera (fig. 1). The reaction was clearly visible at 18 hours and was intense after 2 days. The precipitation line was convex toward the center (precipitin) well, indicating that the antigen is of relatively high molecular weight (Kornfeld et al. 1959). Two intensities of reaction were definable in most groups of sera studied (fig. 2). Some sera lacked detectable antigen, the precipitation lines extending into the corresponding wells without deviation (fig. 1). The precipitation lines were clear-cut and quite unlike the diffuse reaction which slowly develops between rheumatoid factor and \( \gamma \)-globulin in some subjects (Franklin 1960a). Rheumatoid-factor reaction was excluded because neither the patient nor most of the positive subjects had rheumatoid factor, as determined by bentonite-flocculation and latex-fixation tests. Some panel subjects with rheumatoid factor gave reactions with the antiserum, but others did not. A second sample of serum from the patient, drawn 3 weeks after the first, at a time when the patient had not received any medication since discharge, gave identical reactions, as did a third sample drawn 1 month later, and a fourth 3 months later.

Support for the interpretation that the precipitin in the serum of this patient is an antibody came from experiments showing that the active protein is a 7S-\( \gamma \)-globulin. We propose the designation \( \text{Ag(a-)} \) for sera containing the \( \alpha \)-globulin antigen and \( \text{Ag(a-)} \) for sera that do not contain the antigen. The precipitin in the serum of the patient thus has the specificity anti-\( \text{Ag(a-)} \).

Characterisation of Antigen

A panel serum giving a strongly positive precipitation line in the gel-diffusion test was concentrated three times by ultrafiltration and used for immunoelectrophoresis. The antibody well was filled with serum from the patient. A well-defined precipitation line developed between the \( \alpha_2 \) region and the antibody (fig. 3). This result, along with that in the Ouchterlony plates, suggested that the antigen is a high-molecular-weight \( \alpha_2 \)-component. The only \( \alpha_2 \)-macroglobulin so far defined is the 19S-\( \alpha_2 \) component described by Brown et al. (1954) and Wallenius et al. (1957), which appears to be equivalent to the slow-\( \alpha_2 \) component found on starch-gel electrophoresis (Fouliik and Smithies 1958). Purified \( \alpha_2 \)-macroglobulin (Behringwerke, Marburg) in a wide range of concentrations failed to give precipitation with the patient's serum. Furthermore, when an \( \text{Ag(a-)} \) serum was submitted to starch-gel electrophoresis, the slow-\( \alpha_2 \) fraction recovered by the technique of Gordon (1960) failed to show precipitation with the anti-\( \text{Ag(a-)} \) serum. The further identification of the antigen present in the \( \alpha \)-globulin is currently being undertaken.

Characterisation of Antibody

The patient's serum was submitted to immunoelectrophoresis, and a serum giving a strong reaction in the Ouchterlony tests was placed in the side well. Within 18 hours a well-defined precipitation line appeared in the
\( \gamma \)-globulin region (fig. 4). This result, together with the shape of the precipitation curve in the Ouchterlony plate, suggested that the antibody was a relatively low-molecular-weight \( \gamma \)-globulin. This interpretation was confirmed by an experiment in which the 7S- and 19S-\( \gamma \)-globulin components in the patient's serum were separated by density-gradient centrifugation. Precipitation lines in Ouchterlony plates were obtained with low-molecular-weight fractions giving no reaction with immune-rabbit serum against 19S human \( \gamma \)-globulin as described by Franklin (1960b). It can, therefore, be concluded that the antibody is a 7S-\( \gamma \)-globulin. The serum of the patient's daughter showed no reaction when tested against panel antibody is a VS-‘f-globulin. The serum of the patient's daughter gave the same reaction as fresh sera from the same subjects. The reaction in the fresh sera, however, was in some cases stronger and better defined than in the stored sera. Repeated freezing and thawing of the sera up to at least fifteen times did not affect the reaction. Blood-samples were withdrawn twice weekly for 4 weeks from two normal volunteers whose sera reacted strongly with that of the patient. The reaction remained unchanged during this period. Sera withdrawn approximately twice yearly over a 2-year period from six subjects, two of whom reacted and four of whom did not, consistently gave the same reaction as fresh sera from the same subjects. The reaction in the fresh sera, however, was in some cases stronger and better defined than in the stored sera. Repeated freezing and thawing of the sera up to at least fifteen times did not affect the reaction. Blood-samples were withdrawn twice weekly for 4 weeks from two normal volunteers whose sera reacted strongly with that of the patient. The reaction remained unchanged during this period. Sera withdrawn approximately twice yearly over a 2-year period from six subjects, two of whom reacted and four of whom did not, consistently gave the same reaction. Of 2 sera withdrawn in 1955, 1 was a reactor and the other not; the reactions in sera withdrawn from the same subjects in 1959 were unchanged.

Variation in the position of the slow-\( \alpha_2 \)-globulin after starch-gel electrophoresis has been noted. By means of vertical electrophoresis, using borate buffer at pH 8.6 (Smithies 1959), a slow-moving, a fast-moving, and a double band can be distinguished. It has been suggested that this variation may be due to storage (Smithies 1955); but this may not be the only explanation. Sera from different persons stored for the same length of time under identical conditions may show the variation, and not all stored sera show it (Blumberg 1961). In any event, this does not appear to affect the reaction with the patient's serum. Persons of each of the three slow-\( \alpha_2 \)-types were both positive and negative reactors.

**Stability of the Antigen**

The antigen is unaffected by heating at 57°C for 40 minutes, by hemolysis with its own or foreign human hemoglobin, or by storage at room temperature for up to 7 days. The antigen was not lost by dialysis or ultrafiltration. Sera of either type (reacting or non-reacting) stored for as long as 4 years at -20°C were consistently found to give the same reaction as fresh sera from the same subjects. The reaction in the fresh sera, however, was in some cases stronger and better defined than in the stored sera. Repeated freezing and thawing of the sera up to at least fifteen times did not affect the reaction. Blood-samples were withdrawn twice weekly for 4 weeks from two normal volunteers whose sera reacted strongly with that of the patient. The reaction remained unchanged during this period. Sera withdrawn approximately twice yearly over a 2-year period from six subjects, two of whom reacted and four of whom did not, consistently gave the same reaction. Of 2 sera withdrawn in 1955, 1 was a reactor and the other not; the reactions in sera withdrawn from the same subjects in 1959 were unchanged.

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TABLE III—OBSERVED AND EXPECTED NUMBERS OF Ag(a—) CHILDREN FROM VARIOUS MATINGS

<table>
<thead>
<tr>
<th>Test</th>
<th>Mating</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comparison of observed and expected number of Ag(a—) children in families where there is at least one Ag(a—) child</td>
<td>Ag(a+) x Ag(a—)</td>
<td>0.99</td>
</tr>
<tr>
<td>Comparison of observed and expected number of Ag(a—) children in families where there is at least one Ag(a—) child</td>
<td>Ag(a+) x Ag(a+)</td>
<td>1.37</td>
</tr>
<tr>
<td>Comparison of observed and expected number of families with at least one Ag(a—) child, assuming an Ag-gene frequency of 0.66</td>
<td>Ag(a+) x Ag(a—)</td>
<td>0.49</td>
</tr>
<tr>
<td>Comparison of observed and expected number of families with at least one Ag(a—) child, assuming an Ag-gene frequency of 0.66</td>
<td>Ag(a+) x Ag(a+)</td>
<td>0.03</td>
</tr>
<tr>
<td>Comparison of observed and expected number of recessives in Ag(a—) x Ag(a—) matings</td>
<td>Ag(a+) x Ag(a+)</td>
<td>0</td>
</tr>
</tbody>
</table>

\( 2 \chi^2 = 2.88 \) with 5 degrees of freedom; 0.8 > \( p > 0.7 \).

synthesis of the Ag(a+) antigen. If a product of the allelic gene is found, the gene could be termed AgB and the antigen Ag(b+).

The family data were analysed by the methods summarised by Smith (1956). In this, four types of comparison are made, in each of which a correction is included for family size. The comparisons of observed and expected figures involve:

1. The number of recessive Ag(—) children resulting from Ag(+) x Ag(a—) matings, given the number of families in which there is at least one recessive child.
2. The number of recessives resulting from Ag(+) x Ag(a—) matings in which there is at least one recessive offspring, given the total number of families with at least one recessive offspring.
3. In Ag(+) x Ag(a—) matings, the total number of families with at least one recessive offspring, given the total number of Ag(+) x Ag(a—) matings and the gene frequencies in the population from which the families were selected (\( q = \) frequency Ag = 0.66).
4. In Ag(+) x Ag(a+) matings, the total number of families with at least one recessive offspring, given the total number of Ag(a+) x Ag(a+) matings and the gene frequency.

The first two computations are independent of the gene frequencies in the population study. For the third and fourth computations, however, an estimate is needed of the gene frequency in the population from which the families were selected, and the gene frequency was estimated from the frequency of the phenotypes in the parental members of the families (table I). Of 56 parents, 57% were found to be Ag(a+).

The results of these computations are summarised in table III, which includes also the results of Ag(a—) x Ag(a—) matings. Although the numbers are small, this preliminary family analysis is consistent with the genetic hypothesis. On the basis of segregation in this group of families, no evidence of close linkage was found with the following blood-group systems and haptoglobins types: ABO, Rh, MN, Duffy, Kidd, and P. Analyses of 30 twin pairs to be reported elsewhere were also consistent with the genetic hypothesis.

Independence of the Ag and Other Known Inherited Systems of Serum-proteins

The reactions in the panel sera showed that the Ag reactions were independent of rheumatoid factor and of haptoglobin, transferrin, and \( \gamma \)-globulin types. The segregation of the AgA and Ag genes in families was also independent of these systems. Since the Ag(a+) factor is an \( \alpha \)-globulin it is also independent of another recently described inherited \( \gamma \)-globulin system designated In, by Ropartz (1960). In collaboration with Dr. J. Hirschfeld, of Stockholm, Ag tests were carried out on 113 sera of known Gc types, which are distinguishable by immunoelectrophoresis (Hirschfeld and Beckman 1960). Positive and negative Ag reactions were found in subjects of Gc types 1−1, 2−1, and 2−2, the percentage of reaction being of the same order in all these groups. Hence the Ag system is independent of all other known inherited serum-protein differences. The Ag(a+) antibody is also distinct from the factor in normal human serum giving a precipitate in agar with red-cell haemolysates (Peetom et al. 1960); the antigen in the latter case is not present in serum and does not react as an \( \alpha \)-macroglobulin. The Ag factor is present in too many normal sera to be C-reactive protein; which in any case migrates in the \( \gamma \)-region in immunoelectrophoresis (Zach and Zimmermann 1959).

Discussion

The precipitin in the patient's serum defines the presence of a factor in some sera from all human populations so far tested. The antigen is an \( \alpha \)-macroglobulin and the precipitin a 7S-\( \gamma \)-globulin. The presence of the antigen appears to be genetically determined, so that a new polymorphic system in human serum-proteins has been revealed.
Besides being of genetical and anthropological interest, the finding of a specific precipitin in the patient's serum suggests that isoimmunisation by human serum-proteins may occur. Though there is no direct evidence that the precipitin was induced by the many transfusions which the patient received, this would seem to be the most probable explanation of the findings. In all, 61 sera of subjects who had received multiple transfusions have been tested, and 3 of these have given definite precipitation lines with some of the panel sera. The reaction which is the subject of this paper was the strongest, and the specificity of the others, which are different from the Ag system, will be reported later. Sera of forty persons who had not received transfusions failed to give precipitation lines with the panel sera.

Though our patient initially tolerated transfusions well, during the past 2 years he has had pyrexial reactions after most, but not all, transfusions. Whether these reactions take place only when donor serum is Ag(a+), and represent an antigen/antibody reaction, is now being investigated. Other properties of the precipitin will be described elsewhere. The unusual history of this case may be irrelevant, since the other subjects showing precipitins had typical thalassaemia.

Summary
A serum from an anaemic patient who had received approximately 50 transfusions was found to give strong precipitation with sera of some, but not all, normal subjects. It appears that an antigen/antibody reaction is involved. The antigen in some normal subjects is an α₂-globulin, and the antibody in the patient is a 7S-γ-globulin. The antigen appears to be inherited according to simple mendelian rules and its frequency varies in different racial groups.

It is possible, though not proven, that antigen/antibody reactions were responsible for severe post-transfusion reactions in the patient, and similar processes may be involved in other unexplained post-transfusion reactions.

We are indebted to Dr. Fred Stohlman and Dr. Archie McKinney for supplying the serum from the patient; to Dr. Sheldon Dray and Dr. Kurt J. Bloch for valuable help and advice; to Dr. J. Hirschfeld, Stockholm, for collaboration with the Gc tests; to Dr. George A. Silver, Montefiore Hospital, New York, for allowing access to families under his care; to Dr. Arthur G. Steinberg for supplying blood from American Negro families and carrying out the γ-globulin typing; and to Dr. Lyman Crittenden for assistance with the linkage studies. Most of this investigation was undertaken while one of us (A.C.A.) was a visiting scientist at the National Institute of Arthritis and Metabolic Diseases.

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— (1959) ibid. 181, 1203.
ANTIGEN POLYMORPHISM OF A
LOW-DENSITY BETA-LIPOPROTEIN.
ALLOTYPY IN HUMAN SERUM
By Dr. B. S. BLUMBERG, Dr. S. DRAY and
Dr. J. C. ROBINSON
National Institutes of Health, Bethesda, Maryland

It is now known that there are several families of
inherited serum protein variants which constitute
polymorphic systems. These include the haptoglobins, transferrins, \( \gamma \)-globulin (Gm.) groups, group-specific components and others (for review see ref. 2). In such systems variations in the protein structures are inherited in simple Mendelian fashion, and two or more forms of the phenotype are common in the population. Individuals receiving multiple trans-
fusions of blood of compatible red cell type would probably receive some transfusions containing serum proteins of a different phenotype from their own. Although there is no evidence that the known differences in the polymorphic serum protein systems are sufficient to lead to the development of antibodies, it was considered possible that some multiply trans-
fused individuals would develop antibodies against
phenotypes of the known polymorphic systems or against others not yet detected. Such a reaction has been examined in rabbits. Oudin, Dray and Young, and Dubiski et al. have shown that serum proteins from individual rabbits are antigenic in
other rabbits. Isoprecipitins have been found to
react with proteins with \( \alpha \), \( \beta \) and \( \gamma \) mobility in agar gel. As few as three or four intravenous injections of serum can lead to the production of precipitating antibodies (Dray, S., unpublished observations). This phenomenon of individual differences in antigenic speci-
ficity of proteins within a species was called ‘allotypy’ by Oudin.

Allison and Blumberg, using the Ouchterlony
 technique, studied the sera of patients who had received multiple transfusions, in an effort to detect
the presence of antibodies. A precipitating antibody was found in the serum of a patient (Mr. C. de B.) with a refractory anemia of unknown etiology, who had received approximately fifty transfusions during the course of three years. The antibody was a \( 7S \) \( \gamma \)-globulin and it reacted with some but not all human sera to form a well-defined precipitin line.

The protein with which the antibody reacted travelled as an \( \alpha \)-globulin protein (probably of high molecular weight) in agar-gel. Family and twin studies demonstrated that the presence or absence of the reacting \( \alpha \)-globulin is under genetic control. Individuals with a single or double dose of the dominant gene designated \( Ag^+ \) (phenotype \( Ag(a+) \); genotypes \( Ag^+Ag^+; Ag^+Ag^+ \)) reacted; those who were homozy-
gous for the alternate allele \( Ag \) did not react (phenotype \( Ag(a-) \); genotype \( AgAg \)). The polymorphism appears to be independent of the haptoglobins, transferrins, group-specific components, \( \gamma \)-globulin types, and of the rheumatoid factor. It appeared, therefore, that the antibody present in the blood of patient C. de B. defined a previously unknown human
serum polymorphism which was designated the
isoprecipitin \( Ag(a) \) system. However, the \( Ag(a+) \)
protein had not been further identified.

In the present article evidence is presented that the
Ag(a+) protein is a low-density β-lipoprotein. (Lipoproteins are classified as low or high density on the basis of their flotation or sedimentation in an ultracentrifuge. The low-density lipoprotein migrates as a β-globulin on paper and an α-globulin on agar-gel electrophoresis; it is usually referred to as the low-density β-lipoprotein.)

The gel-diffusion precipitation technique (Ouchterlony procedure) was carried out in 5 cm Petri dishes containing 8 ml of 0-9 per cent Oxoid 'Jonagar' (w/v) in 0-07 sodium phosphate buffer, pH 7-0. The method used is described elsewhere. Immunoelectrophoresis was done by the method of Grabar and Williams and Scheidegger, using standard lantern slide plates coated with agar made up in barbitone buffer, 0-01 M, pH 8-4. Staining was done by a method modified from that described by Uriel and Grabar. Sudan black B (National Aniline) was used for the lipoprotein and azocarmine (National Aniline) for the protein stain. Single-dimension starch-gel electrophoresis was by the method of Smithies using the tris-(hydroxymethyl)-aminomethane discontinuous buffer system of Poulik and filter paper insertion. Two-dimensional starch-gel electrophoresis was by the method of Poulik and Smithies, in which the first dimension was on paper using an acetate-barbital buffer (3-74 gm. sodium acetate and 11-04 gm. sodium barbital in 1 litre of distilled water) and the second dimension was in gel using the tris buffer. Agar-gel electrophoresis was performed on standard lantern slides using the same conditions as for immunoelectrophoresis. The isolation of γ-globulin was by the method of Levy and Sober. The low- and high-density lipoproteins were separated from each other by ultracentrifugation in a high-density salt medium using whole serum and the method of Havel et al. Two fractions were separated, one of a density lower than 1-063 (low-density lipoprotein) and the second greater than 1-063 (high-density).

Fig. 1 is a photograph of an Ouchterlony experiment before and after staining with sudan black. It was designed to show that the precipitin line formed between the serum of Mr. C. de B. (well 1) and an Ag(a+) individual (centre well) stains with sudan black. A positive control was obtained by reacting rabbit anti-β-lipoprotein (well 6) with the serum of Mr. C. de B. and the Ag(a+) individual; the two precipitin lines coalesce. Two precipitin lines are formed, one with alanine buffer and the other with soybean black. The longer arrow shows the position of the origin, and the broken arrow, the position of the single lipoprotein band. Amido black stain anti-human serum (well 3) and the Ag(a+) serum (centre well) served as another positive control. Two precipitin lines were used for negative controls: (1) between horse anti-human serum (well 3) and isolated α-macroglobulin (which contains no lipoprotein) (well 2); and (2) between horse anti-γ-globulin (well 5) and the Ag(a+) serum (centre well). Neither of these lines stained with sudan black.

Fig. 2 is a photograph of an Ouchterlony plate designed to compare the reactions of low- and high-density lipoproteins prepared from the serum of Ag(a+) and Ag(a-) individuals. Mr. C. de B.'s serum reacts only with the whole serum and the low-density β-lipoprotein of the Ag(a+) serum but not with the high-density lipoprotein. As expected,
Mr. C. de B.'s serum does not react with the Ag(a-) whole serum or the fractions thereof. The sera of 8 other Ag(a+) and 8 other Ag(a-) individuals were tested in the same way and the same results as mentioned here were obtained.

Fig. 3 is a photograph of the results of a starch-gel electrophoresis experiment which compares whole serum and the low-density β-lipoprotein fraction from the serum of the same individual. The lipoprotein was approximately three times its concentration in whole serum. It migrates a single well-defined band (broken arrow).

Fig. 4 is a photograph of an immunoelectrophoresis experiment designed to show the reaction between the concentrated 7S γ-globulin (isolated from the serum of Mr. C. de B.) with whole serum, and with high- and low-density lipoprotein. As seen in Fig. 2, the whole serum and the low-density lipoprotein from an Ag(a+) individual reacted to give precipitin lines, but the high-density lipoprotein did not. (As in the Ouchterlony plates, the precipitin lines stained intensely with sudan black (not shown).) Horse anti-human serum also reacted with the whole serum and high- and low-density lipoproteins. By comparison with the horse anti-human serum, it is seen that the low density β-lipoprotein migrates in the agar gel with the electrophoretic mobility of an α-globulin as previously reported.

The experiments summarized here have shown that the low- but not the high-density ultracentrifuge fraction of the serum of an Ag(a+) individual precipitates in agar gel with the 7S β-globulin antibody of Mr. C. de B. (Fig. 2). The precipitin line stains with the lipid stain, sudan black B; it coalesces with a precipitin line found in rabbit anti-human β-lipoprotein and human serum (Fig. 1). The antigen has the mobility of an α-globulin on agar gel (Fig. 4) and migrates as a single band between the origin and the slow α-two globulin on starch-gel electrophoresis (Fig. 3). These experiments identify the antigen as the low-density β-lipoprotein.

The whole serum, low-density and high-density fractions of 45 Ag(a+) and 35 Ag(a-) subjects were analysed for total cholesterol, total phospholipid and the total cholesterol/phospholipid ratio. (We are indebted to Dr. U. S. Fredrickson for supplying these data.) There were no significant differences in these broad lipid classes between the Ag(a+) and Ag(a-) individuals. It would be of interest if chemical differences in the β-lipoproteins of Ag(a+) and Ag(a-) individuals could be found.

In an experiment designed for other purposes, two chimpanzees were immunized with a single injection of whole human serum of an Ag(a-) individual plus complete Freund's adjuvant. Serum was obtained four weeks later. Both the animals developed precipitating antibody to human low-density β-lipoprotein as evidenced by a single band in agar gel. The chimpanzee antibody reacted with 225 normal American human sera tested and did not distinguish between Ag(a+) and Ag(a-) individuals. When the chimpanzee sera were used instead of rabbit anti-β-lipoprotein serum, coalescence was seen between the three precipitin lines similar to that observed among the centre, first and sixth wells of Fig. 1. Further immunization experiments with lower primates are in progress. We hope to obtain antibody which will distinguish individual differences in human β-lipoproteins.

This article, in conjunction with the genetic data presented elsewhere, establishes that a genetic polymorphism exists in the human low-density β-lipoprotein. The factors which maintain this polymorphism are unknown, although they may be related to diseases in which the lipoproteins are involved, such as some kinds of arteriosclerosis, diabetes and coronary heart disease. It would be of interest to determine if antigenic polymorphisms of β-lipoproteins (allotypy) occur in species other than man; for example, in rabbits, where allotypy for the α- and β-globulin has already been reported.
4 Dray, S., and Young, G. O., J. Immunol., 81, 142 (1958); Science, 129, 1023 (1959); ibid., 131, 738 (1960).
5 Dubiski, S., et al., Immunology, 2, 85 (1959).
POLYMORPHISMS OF THE SERUM
PROTEINS AND THE DEVELOPMENT OF
ISO-PRECIPITINS IN TRANSFUSED
PATIENTS*

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National Institutes of Health, Bethesda, Maryland

Polymorphism, according to Ford,¹ is the occurrence in
the same habitat of two or more inherited forms of a
species in such proportions that the rarest of them can¬
not be maintained merely by recurrent mutation. This
definition excludes the rare, genetically disadvantageous
inherited diseases which are maintained by mutation and selected against
by death or infertility. In polymorphic traits two or more of the geno¬
types determining variation of the trait are common in the population.
Although much of the early work on polymorphism was performed
on lower animals, it is now clear that humans constitute one of the
most favorable species in which to study these systems. In addition,
the polymorphisms provide convenient systems for the study of in¬
herited discontinuous biochemical variation in man.

Polymorphisms are thought to arise as a result of selective differ¬
ences between genotypes. For example, the sickle cell homozygote
develops a severe hemolytic anemia which, under natural conditions,
is usually fatal. The heterozygote, however, is at a selective advantage
compared to the normal homozygote, apparently because of greater
resistance to falciparum malaria, and increased fertility. As a consequence
of the selection in favor of the heterozygote, the sickle gene is main¬
tained in the population at high levels despite the elimination of genes
due to the death of the homozygotes.² Other examples of associations
of polymorphisms with exogenous disease-producing agents are known.
Individuals bearing the sex-linked glucose-6-phosphate dehydrogenase
deficiency (G6PD) gene (and probably at least one other trait as well)

presented at a combined meeting of the New York Rheumatism Association and the Section on
Medicine, held at The New York Academy of Medicine, November 18, 1963.
can develop a hemolytic anemia when they eat, or are otherwise exposed to the fava bean. A similar anemia may develop if they ingest any of a large number of drugs. Individuals without this trait will not develop the anemia given the same exposure. Similarly, individuals with the inherited atypical serum cholinesterase trait can develop prolonged apnea after the administration of Suxamethonium or other related drugs (see below). It is probable that disease susceptibility related to a single inherited trait is less common than susceptibility dependent on several inherited traits. The identification of such traits may serve a useful function in identifying individuals who require special protection against specific exogenous agents.

As a consequence of disease and environmental forces, as well as other factors, a large number of polymorphisms may exist in a population. Some may be related to present selective forces, and others to forces which operated in the past, but which are no longer significant. Present gene frequencies may also result from gene mixture between populations.\textsuperscript{8}

Table I is a list of some of the biochemical polymorphisms known in man. In the present lecture I would like to discuss the polymor-

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**Table I.—SOME POLYMORPHIC TRAITS IN HUMANS**

<table>
<thead>
<tr>
<th>Red Blood Cell Types</th>
<th>Serum Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABO</td>
<td>Haptoglobin</td>
</tr>
<tr>
<td>MNS</td>
<td>Transferrin</td>
</tr>
<tr>
<td>P</td>
<td>Gamma globulin</td>
</tr>
<tr>
<td>Rhesus</td>
<td>Beta-Lipoprotein</td>
</tr>
<tr>
<td>Lutheran</td>
<td>Group-specific substance</td>
</tr>
<tr>
<td>Kell</td>
<td>Serum cholinesterase</td>
</tr>
<tr>
<td>Lewis</td>
<td>Serum esterase</td>
</tr>
<tr>
<td>Duffy</td>
<td></td>
</tr>
<tr>
<td>Kidd</td>
<td></td>
</tr>
<tr>
<td>Diego</td>
<td></td>
</tr>
<tr>
<td>Sutter</td>
<td></td>
</tr>
<tr>
<td>X-linked</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other Cell Types</th>
<th>Miscellaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobins</td>
<td>RAB urinary excretion</td>
</tr>
<tr>
<td>G6PD</td>
<td>Secretor of ABH</td>
</tr>
<tr>
<td>Red cell phosphatase</td>
<td>blood group substance</td>
</tr>
<tr>
<td>Red cell phosphogluconate dehydrogenase</td>
<td>Taste of PTC</td>
</tr>
<tr>
<td>White blood cell antigens</td>
<td>Ear cerumen character</td>
</tr>
<tr>
<td>Platelet antigens</td>
<td></td>
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</tbody>
</table>

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Serum Protein Polymorphisms (Figure 1)

The haptoglobins. The haptoglobins are a family of serum proteins which bind hemoglobin. The early studies of Jayle and his co-workers demonstrated that the haptoglobins are elevated in a wide variety of inflammatory diseases. In some illnesses associated with hemolysis, the hemoglobin released from the breakdown of the red blood cells combines with the haptoglobin to form a strong complex which appears to be removed mainly by the reticuloendothelial system. If the rate of breakdown of red blood cells is greater than the production of haptoglobin, the serum haptoglobin level will decrease, in some cases, to undetectable levels. If the hemolytic process is arrested, the haptoglobin
will be regenerated, presumably in the liver, and the serum levels may return to normal.

When starch gel is used as a supporting medium for electrophoresis, human sera can be separated according to haptoglobin type. Three major types, 1-1, 2-2, and 2-1, can be distinguished. It has been shown that in most populations these are controlled by a pair of allelic autosomal genes designated $Hp^1$ and $Hp^2$. $Hp^1$ homozygotes are type 1-1, $Hp^2$ homozygotes are type 2-2, and heterozygotes are type 2-1. Recently, Nance and Smithies have shown that when purified serum haptoglobin is subjected to reductive cleavage by mercaptoethanol in the presence of 8 M urea, fast-moving and slow-moving haptoglobin variants can be distinguished. By chemical and genetic studies they demonstrated that there are three genetically determined alpha polypeptide chain variants controlled by five allelic genes $Hp^1F$, $Hp^1S$, $Hp^2F$, $Hp^2S$, and $Hp^{2SS}$. There are other relatively rare genes (i.e., $Hp^{2M}$) which control uncommon haptoglobin phenotypes.

Allison, Blumberg, and apRees reported that approximately 30 percent of a West African population had insufficient haptoglobin to permit typing by the starch gel method. Since this original description a-haptoglobinemia and hypohaptoglobinemia have been detected in other Africans, in U. S. Negros, and in some European and Asiatic populations. There is good evidence that some forms of a-haptoglobinemia or hypohaptoglobinemia are inherited, but some cases are due to the hemolysis of red blood cells and subsequent loss of haptoglobin in a variety of hemolytic diseases.

The transferrins. The transferrins (or siderophilins) are a group of serum proteins which bind iron. They can be identified by their mobility in starch gel electrophoresis and by the use of radioactive iron and autoradiography. The phenotypes are controlled by a series of apparently allelic genes, $Tf^C$, $Tf^{BO}$, $Tf^{B1}$, etc. The most common phenotype in U. S. and European population is type C, which corresponds to the genotype $Tf^C/Tf^C$. The slow-moving D variants have been found in African, American Negro, Australian aboriginal, Chinese, and other populations. Fast-moving types are occasionally found in white, Asiatic, and other populations. In recent years a large number of variants with only minute differences in electrophoretic mobility have been detected. To date no associations with particular illnesses have been found, and there does not appear to be a significant difference in the amount of
iron bound by each of the phenotypes. It has been stated that the transferrins have an inhibitory effect on virus multiplication, but it is not clear if this effect has any biological importance.

_Gamma globulin groups._ Inherited differences in the gamma globulins were detected as a result of a chance observation made while using an immunological test for rheumatoid arthritis. The serum of many patients with rheumatoid arthritis has a gamma globulin of high molecular weight called “rheumatoid factor,” which agglutinates blood cells or other particles coated with specially prepared serum gamma globulin. Grubb used a system in which Rh- human red blood cells were coated with an incomplete anti-Rh antibody. Some sera of patients with rheumatoid arthritis would agglutinate these particles. Grubb found that a gamma globulin present in the serum of some, but not all, normal individuals could inhibit the agglutination reaction. The inhibiting material was inherited in simple Mendelian fashion. Individuals homozygous or heterozygous for a dominant gene Gm were inhibitors. Those homozygous for all alternate allele Gm did not have inhibiting material in their serum. Using different combinations of coated red blood cells and sera of rheumatoid arthritis patients, different genetically controlled gamma globulins have been detected. Several of them are controlled by a series of allelic genes at the same locus (GmA, GmB, GmC, etc.), and others by genes at a second locus (InvA, InvB). There is as yet no information on the nature of the physiological or pathological conditions associated with this trait, although it may be connected in some way with the immune process.

_Genetic variations of human serum phosphatase._ Using starch gel electrophoresis, Arfors et al. have shown that there are at least two types of human serum phosphatases. All human sera have one phosphatase band which migrates with a mobility slightly slower than serum transferrin. Some individuals, in addition, have a slower moving phosphatase band. On this basis, human sera were classified in two groups: Group I with a single phosphatase band and group II with two bands. Twin studies were highly consistent with the hypothesis that these types are inherited, but the mode of inheritance is not known. The authors also found a strong association with the Lewis blood group system, and they suggested that one serum phosphatase zone may represent the Le substance or a complex with the Le substance.

_Polymorphism of α1-acid glycoprotein._ Alpha1-acid glycoprotein is
a serum protein with a high (40%) carbohydrate content. It corresponds with the slow pre-albumin band on starch gel electrophoresis (Fig. 1). Schmidt and his associates\textsuperscript{10} developed a technique for isolating large quantities of this protein from human serum or plasma. The isolated material was concentrated and subjected to starch gel electrophoresis. In comparing material isolated from different individuals, they found different patterns containing varying numbers of bands. Preliminary twin studies indicate that these patterns are inherited.

\textit{Group-specific components.} Hirschfeld\textsuperscript{11} has described a variation in the serum $\alpha$-globulin which is detected by immunoelectrophoresis and is inherited in simple Mendelian fashion. The $\alpha_2$-globulin involved is in the postalbumin region on starch gel electrophoresis.\textsuperscript{12} Differences in mobility of these bands were noted by Smithies in his early studies using vertical starch gel electrophoresis. No associations with disease have been detected using this system.

\textit{Serum cholinesterase.}\textsuperscript{13} There is a gene (whose frequency in the populations tested is approximately 0.02) which determines the presence of an atypical serum cholinesterase. Individuals who are homozygous for this gene have only the atypical enzyme and are unable to inactivate the muscle relaxant succinylcholine (Suxamethonium) and related drugs with the same rapidity as individuals who are homozygous for the normal gene. When succinylcholine was administered to individuals homozygous for the atypical gene, they developed a prolonged apnea. Heterozygotes have both typical and atypical enzymes. Since the homozygotes are easily identifiable by means of a simple spectrophotometric test requiring less than 0.1 ml. of serum, it is quite practical to pre-test individuals receiving muscle relaxants. This is particularly important in shock therapy where patients often receive a prolonged course of muscle relaxants during their treatment. Other drugs which probably are also differentially metabolized by the typical and atypical enzyme include procaine hydrochloride (Novocain), phystostigmine (Eserine), and chlorpromazine. It is, of course, unlikely that the differences in drug susceptibility are responsible for the maintenance of the polymorphism. However, a number of solanaceous plants contain an inhibitor of cholinesterase. Potato peels, for instance, contain an inhibitor which can distinguish between the three cholinesterase phenotypes. It should be emphasized that under normal conditions the atypical enzyme homozygotes are quite normal; they can only be distinguished from the other

\textsuperscript{Bull. N. Y. Acad. Med.}
Isoantibodies in transfused patients. The existence of many polymorphisms of serum proteins makes it highly likely that a transfused patient would receive serum proteins slightly different from his own. For example, an individual of haptoglobin type 1-1, which occurs in about 15 per cent of the normal U. S. white population, has 85 of 100 chances of receiving plasma containing haptoglobin -2, if the donor is also U. S. white. Similar possibilities of receiving a different protein occur with many of the other systems. If this is so, then multiply transfused patients would often receive plasma containing “foreign” protein, albeit from the same species. In a study of transfused sera using the relatively insensitive technique of double diffusion in agar gel, a serum from a patient (C. de B.) was found to contain a precipitin which reacted with a protein present in the serum of some but not all normal individuals. The isoprecipitin did not react with any of the known polymorphic proteins, but was directed against a low density beta-lipoprotein. This technique revealed a “new” polymorphic system.

The ability of a serum to show a precipitin reaction with the human anti-human antiserum was found to be inherited. Individuals homozygous or heterozygous for a gene $Ag^A$ reacted with antiserum C. de B. and individuals homozygous for its alternate recessive allele did not react. Further studies were initiated using the sera of other transfused patients and more than 20 sera containing isoprecipitins were found. In a study of 11 of these, at least 5 and probably 7 different specificities were identified. Preliminary family studies with two of
these suggest that they are inherited, and it is likely that the other specificities are inherited as well. Most of the antigenic specificities so far studied are common in most populations (see Table II). It appears that most individuals have several inherited antigenic specificities on these lipoproteins.

The isoprecipitins against serum lipoproteins are quite common in multiply transfused individuals. Approximately one third of thalassemia patients and 10 per cent of other patients who receive 35 or more transfusions will develop detectable isoprecipitins. The reasons for the higher frequency in thalassemia patients are not clear.

Patients who develop an isoantibody usually require additional transfusions, and this could presumably lead to antigen-antibody interactions in vivo similar to those studied in animals. Approximately 15 patients have been observed for varying lengths of time after the isoprecipitin was discovered. In all cases, the patients have needed frequent transfusions, very often with blood known to contain the proteins which in vitro form precipitin bands with the patient's isoprecipitin. In none of these cases has it been possible to associate with the isoprecipitin an acute, severe transfusion reaction. It has not been possible as yet to evaluate the long-term effect of continued transfusions.

The selective or disease factors, if any, associated with this polymorphism are unknown. As a preliminary investigation of possible

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**Table III.**

<table>
<thead>
<tr>
<th></th>
<th>C. de B.</th>
<th>New York</th>
<th>J. B.</th>
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<tr>
<td></td>
<td>Total</td>
<td>% Reactors ?</td>
<td>Total</td>
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<td>Coronary Artery Disease</td>
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<td>Rheumatic Fever</td>
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<tr>
<td>Control</td>
<td>152</td>
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Reactions of three anti-lipoprotein antisera with sera from diseased patients and controls. The coronary artery disease and rheumatic fever patients and their controls were U. S. Whites and the diabetes patients and their controls were U. S. Negroes. The number of sera which could not be typed are shown in the column headed by a question mark. These studies are described in greater detail elsewhere.
disease associations, two entities known to be associated with abnormalities of the lipoproteins were studied. In addition, the sera of patients with rheumatic fever, collected for other reasons, were also studied. The frequency of reaction of these patients and controls with three of the antisera are shown in Table III.\textsuperscript{17} There is a significantly higher number of reactors with the C, de B, antisera in the diabetic as compared to the control group. No such differences are detected for the other groups. This finding is interesting, but before any importance can be attached to it, this study will have to be repeated in other populations. If it proves to be reproducible, then the higher frequency of reactors might be due to some feature of the disease which converts apparent non-reactors into detectable reactors, or it may possibly be related to an inherited susceptibility factor.

In addition to the isoprecipitins which react with lipoproteins, an isoprecipitin has been found which reacts with an as yet unidentified protein which does not appear to have a large amount (or in some cases, any) lipid. The isoprecipitins are found in multiply transfused hemophilia patients. The protein with which they react is very rare in populations of Western origin, but it is not uncommon in Micronesians, Vietnamese, Formosans and Australian aborigines. The reacting antigen is also quite common in patients with leukemia, and the significance of this is currently under study.

In addition to these precipitating antibodies, Allen and Kunke\textsuperscript{18} have found agglutinating antibodies against gamma globulin (anti-Gm antibodies) in the sera of many multiply transfused thalassemia patients. It is not unlikely that, using the appropriate techniques, antibodies against a very large number of serum proteins will be detected. These may be the cause of some of the transfusion reactions which cannot be ascribed to interaction with the formed elements of the blood.

**Summary**

The concept of polymorphism is discussed and known biochemical polymorphisms in man are listed. The serum protein polymorphisms and the formation of isoprecipitins in multiply transfused human patients are described.

**References**

2. Allison, A. C. Aspects of polymorphism...


Albumin Naskapi: A New Variant of Serum Albumin

Abstract. An apparently new variant of human serum albumin, albumin Naskapi, has been found in high frequency in the Naskapi Indians of Quebec and, in lower frequency, in other North American Indians. The family and population data of the albumin are consistent with its inheritance as a simple autosomal trait controlled by a gene designated Al Naskapi. This gene is allelic with the gene A1a which controls the common albumin. Both homozygotes and heterozygotes have been distinguished. This is the first report of a homozygote for an albumin variant.

Rare electrophoretic variants of serum albumin have been reported several times since the original description of bisalbumin by Scheurlen (1). In nearly all of the reports, the rare variant has an electrophoretic mobility less than that of the common form of albumin (1-4), but two investigators reported fast-moving, electrophoretic variants of albumin which is different from at least one of the other fast-moving variants (5, 6). The trait is common in several North American Indian tribes, but has not been seen in other populations tested. Several homozyogotes for the variant have also been identified.

The original finding of the albumin variant, and the subsequent screening, was made with whole serum with Ashton's discontinuous buffer system at pH 8.6 (7). The gels were prepared from hydrolyzed starch (Connaught) at a concentration 25 percent higher than that recommended by the manufacturers. The electrophoresis was performed in a horizontal system with a constant voltage of 9 volt/cm. The discontinuous buffer system (pH 8.6) described by Poulik (8) and Ashton's acid buffer system (pH 5.6) (9) with a constant voltage of 16 volt/cm were also used.

Cellulose acetate electrophoresis was performed with a microzone electrophoresis system in barbital buffer at pH 8.6. Immunoelectrophoresis was done in 1 percent special agar-noble gel with a modified barbital buffer (10) and horse antiserum to human albumin. Barbital buffer (pH 8.6; ionic strength, 0.75) was used in the paper electrophoresis experiments.

The populations studied are shown in Table 1. In several cases, the blood specimens were used in other studies, and the populations are described in the indicated references. Blood was collected by venepuncture, and the serum or plasma was separated and stored at $-20^\circ$C until tested. The dates of collection vary from 1958 for the Athabascans to 1962 for the Naskapi and Montagnais Indians.

The fast-moving variant can be distinguished by electrophoresis in starch gel with Ashton's discontinuous buffer, Ashton's acid buffer, or cellulose acetate. It can also be seen with Poulik's buffer, but only when the serum is diluted. Good separations were not obtained with barbital buffer with paper as a supporting medium. The three phenotypes can be easily distinguished (Fig. 1). In immunoelectrophoresis the homozygote has a slightly elongated albumin band, and the new fast homozygote has a greater mobility than the common slow homozygote. However, antigenic differences between the two albumins are not seen. In Fig. 1, the new variant is compared to that described by Wieme (6); it is clearly different. The fast-moving variant described by Tarnoky and Lestas (5) was not available for comparison.

The distribution of the trait in several populations and the expected frequencies as calculated by the Hardy-Weinberg formula are shown in Table 1. Serum from 365 Haida Indians from Canada (12), 100 Quechua and 92 Cashinahua Indians from Peru (13), 443 Eskimos from Alaska (12), and 114 Americans of European descent were tested. None of these had albumin Naskapi. The results correlate well with the expected distribution. The gene frequency of the new variant in the Indian populations ranges from 0.13 in the Naskapi to 0 in the Haida and South American groups. None was seen
in the Eskimos or Caucasian Americans tested. Many of the smaller populations of Indians included related individuals, a fact which may affect the calculation of the gene frequency. Therefore, the frequencies given in Table 1 are only approximate. Three pedigrees in which the trait is segregating are shown in Fig. 2. The first (Naskapi) shows the segregation of the trait in three generations; the albumin types of all parents are known. In the second (Montagnais), a mother with both types of albumin has three children, of which one has only the common albumin, one has only the new variant, and the third has both forms. The third (Naskapi) shows a large family where the variant apparently has segregated to five of seven children in the oldest generation. A man in the second generation has only the new type, while his three children from two marriages have both types. An analysis of eleven Naskapi and Montagnais families (including some not in the pedigrees of Fig. 1) is shown in Table 2.

The albumin is different from the slow-moving variant and from the fast-moving albumin described by Wieme (6). It is proposed that this new variant be called albumin Naskapi, after the Indian population in which it was first discovered, and that the gene determining the trait be designated Al Naskapi (abbreviated Al\textsuperscript{N}). The common albumin variant is designated albumin A (gene notation Al\textsuperscript{A}) and the originally described slow-moving variant, albumin B (gene notation Al\textsuperscript{B}). As new variants are discovered, it would be convenient to give them geographic, population, or other specific designations.

The segregation of Al Naskapi in the families is consistent with simple autosomal inheritance, with full expression of Al Naskapi and the alternate gene Al\textsuperscript{A} in the heterozygote. On the basis of the populations tested, albumin Naskapi appears to be restricted to Northern American Indians. The high frequency in the Naskapi could be due to the operation of selective forces in the environment of these populations, or to genetic drift.

The Naskapi and Montagnais have a high frequency of two transferrin variants which are rare in most other populations (14). The transferrins are not found in any of the other Northern Indian groups, with the exception of the Tlingit, where they are found in very low frequency. If the high frequencies of albumin Naskapi in the Indians were due to simple gene mixture, and if selection were not operating on either of these protein polymorphic traits, then it would be expected that the transferrin variants would occur in the same Indian groups in which the albumin variant occurs. This was not found to be the case.

There appears to be a broad north-south cline for the frequency of albumin Naskapi which cuts across known ethnic relations of the Indians. This is the reverse of the cline for the genes which determine the Diego factor and haptoglobin-1, both of which occur with low frequency in North American tribes but in high frequency in Central and South American tribes. In addition to the albumin Naskapi, we have found an albumin variant which occurs with high frequency in the Eskimo population of a small Indian Eskimo village in Alaska. This variant may be different from albumin Naskapi, but additional specimens will be needed to complete the comparison. A slow-moving variant occurs with

<table>
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<th>A/Naskapi (No.)</th>
<th>A/A (No.)</th>
<th>Gene frequency Al\textsuperscript{B}</th>
<th>Al\textsuperscript{A}</th>
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<td>16.5</td>
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<tr>
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<td>0.007</td>
<td>0.993</td>
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<td><strong>Ten families, A/A - A/Naskapi</strong></td>
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<td>17</td>
<td>15</td>
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<tr>
<td>16</td>
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</table>

Table 1. Distribution of albumin types in different populations.

Table 2. Segregation of albumin types in families.
relatively high frequency in Mexican Indians. Therefore, our findings indicate that albumin variants occur with greater frequency than previous evidence had indicated.

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References and Notes
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5 July 1966
THE GENETICS OF HUMAN SERUM ALBUMIN

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I. INTRODUCTION

A. Historical Review

The study of the genetics of human serum albumin began with Scheurlen's report in 1955 of a diabetic female whose serum on paper electrophoresis had a slow-moving albumin band in addition to the common one. Subsequently, Wuhrmann reported that the two albumin bands were present in the patient irrespective of her clinical status and that two albumins, one slow, the other of the usual mobility, were present in the sera of her father, brother and son. This suggested that the double albumin was an inherited trait. The distribution of the variants in all the families that were tested was consistent with a codominant mode of inheritance, each allele controlling the synthesis of each albumin fraction.

†This work was supported by USPHS Grants CA-06551, RR-05539 and CA-06927 from the National Institutes of Health, and by an appropriation from the Commonwealth of Pennsylvania.
The genetic hypothesis gained support as additional cases of uncommon albumin bands were reported. Slow-moving variants were found in Europeans from several countries including Germany, the United States, France, Italy, the United Kingdom, Sweden, Norway and Denmark. These slow albums all appeared to have the same electrophoretic mobility and were termed albumin B. The common more rapidly moving band was designated albumin A. Two fast-moving albumin bands were reported in families of European origin, albumin Gent from a Belgian family and albumin Reading from a Welsh/English family. These variants were rare in the populations in which they were found (in the order of one in several thousand) and seem to be examples of mutations at the albumin locus which had not increased to high frequencies in any of the populations surveyed.

In 1966 Melartin and Blumberg discovered a fast albumin variant among American Indians. It differed significantly from those previously reported not only in its mobility but because it occurred relatively commonly (several per hundred) in the populations in which it had been detected. It was found originally in the Naskapi and Montagnais Indians of Quebec Province, Canada, and was termed albumin Naskapi. Some Naskapi and Montagnais Indians lacked the common albumin A and had only the fast-moving albumin band in their sera. This was the first report of individuals apparently homozygous for an albumin other than the common one. In 1967 another case of a single fast-moving albumin band was reported in sera from an Indian family living in Alberta, Canada, and this was subsequently shown to have the same electrophoretic mobility as albumin Naskapi. In the same year a new slow-moving variant, albumin Mexico, was discovered among Indians of Mexico. This also was relatively common among the Indian populations in which it occurred.

By 1967 the numerous family studies, electrophoretic comparisons and the reports of apparent homozygotes confirmed the earlier suggestions of genetic control of electrophoretic variation of albumin. Cases of transient double albumins have also been reported. There is no evidence that these are under genetic control.

When the first review of what had then become a sizable literature on albumin variation was published by Melartin in 1967 there were only a half-dozen albumin variants. The number has since increased about fourfold. The rapid increase is primarily due to the application of relatively simple screening techniques to sera from South America and Asia. New variants have been found among Japanese, Malaysians, Indonesians, Indo-Dravidians, New Guinea aborigines and South American Indians.

The distribution of albumin variation appears to be worldwide. The only exception is sub-Saharan Africa, although this may be due to the paucity of observations. To date only two cases of alloalbuminemia in individuals of African ancestry have been reported.

B. Terminology

There are now over two dozen albumin variants. Originally, the term bisalbuminemia (from the Greek, meaning two albumins in the blood) was used to describe the presence of two types of albumin in the sera (i.e. the heterozygote). When homozygotes were discovered, the term bisalbuminemia became inaccurate.
The condition, alloalbuminemia, is recognized by the presence of an albumin band which has an electrophoretic mobility different from normal albumin. When a new variant is identified, it is often conventionally named for the population in which it was first discovered.

Melartin suggested that a nomenclature system analogous to that used for transferrins, hemoglobin and other human polymorphic systems be adopted for the albumins. The phenotype of the common albumin is designated Al A (gene symbol $A^A$), and the common genotype is $A^A/A^A$. Variant phenotypes and genotypes are similarly identified but with the variant name or abbreviation in the appropriate script. For example, Al Na is the phenotype for an albumin Naskapi homozygote and $A^N/A^N$ for the genotype.

II. LABORATORY STUDIES

A. The Relative Percentages of Albumin Variants in the Serum

The variant type usually comprises about half of the total albumin whether the variant is fast or slow. There appears to be one exception to this. A very low quantity of the variant fraction (albumin Maku) was reported by Weitkamp and Chagnon.

B. The Antigenic Specificity of Albumin Variants

The antigenic specificity of albumin variants has been tested by Ouchterlony techniques, immunoelectrophoresis against anti-whole human serum, and immunoelectrophoresis against horse, goat, sheep and rabbit anti-human albumin serum. In general, these tests indicate that albumin variants are antigenically identical to normal albumin. There are, however, two exceptions. Robbins et al. noted in a report of an unnamed variant from Utah that during immunoelectrophoresis with undiluted serum one precipitin arc was formed, but when the variant serum was diluted (1:30) prior to immunoelectrophoresis, two overlapping precipitin arcs were formed. Margni et al. has also noted (using absorption studies) slightly different antigenic properties between sera from two alloalbuminemic families.

C. Electrophoretic Mobilities of Albumin Variants

Variants of human serum albumin have been recognized by differences in their electrophoretic mobility. Such differences would be found only if the amino acids which distinguish the variants carry an electric charge. However, an albumin molecule with a variant amino acid composition may not be detected if the distinguishing amino
<table>
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<th>Notes</th>
<th>Reference</th>
<th>Provenance/ethnic origin</th>
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<td>A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Slow: Same mobility as &quot;Kashmir&quot; according to Weitkamp et al.&lt;sup&gt;64&lt;/sup&gt; &quot;Kashmir&quot; is formally precedent</td>
<td>Knedel&lt;sup&gt;67&lt;/sup&gt;</td>
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<td>Afghanistan</td>
<td>May be same as &quot;Mexico&quot; of Melartin &amp; Blumberg&lt;sup&gt;65&lt;/sup&gt;</td>
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<td>Algonkian</td>
<td>Subsequently found identical to &quot;Naskapi&quot;&lt;sup&gt;144&lt;/sup&gt; &quot;Naskapi&quot; is formally precedent</td>
<td>Polesky &amp; Rokala&lt;sup&gt;145&lt;/sup&gt;</td>
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<td>Emanuelle et al.&lt;sup&gt;148&lt;/sup&gt;</td>
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<td>Earle et al.&lt;sup&gt;151&lt;/sup&gt;</td>
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<td>Lau et al.&lt;sup&gt;171&lt;/sup&gt;</td>
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L. M. SCHELL AND B. S. BLUMBERG
<table>
<thead>
<tr>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuala Lumpur</td>
<td>Slow</td>
</tr>
<tr>
<td>Kyoto</td>
<td>Diner; same as &quot;Makiritare-1&quot; and &quot;Piaroa&quot;</td>
</tr>
<tr>
<td>Larson</td>
<td>Diner; same as &quot;Makiritare-1&quot;, &quot;Piaroa&quot;</td>
</tr>
<tr>
<td>Luarca</td>
<td>Same as &quot;Makiritare-1&quot;, &quot;Belem III&quot;, &quot;Maku&quot; is formally precedent</td>
</tr>
<tr>
<td>Makiritare</td>
<td>Tanis &amp; Neel (unpublished)</td>
</tr>
<tr>
<td>Makiritare-1</td>
<td>See &quot;Makiritare-2&quot;, &quot;Belem III&quot;</td>
</tr>
<tr>
<td>Makiritare-2</td>
<td>Probably same as &quot;New Guinea&quot;</td>
</tr>
<tr>
<td>Maku</td>
<td>A polymorphic variant may be same &quot;Belem II&quot; or &quot;P&quot; of Polesky et al.</td>
</tr>
<tr>
<td>Maprik</td>
<td>May be &quot;SO/BS&quot;</td>
</tr>
<tr>
<td>Mexico</td>
<td>A polymorphic variant; same as &quot;Algonkian&quot; of Polesky &amp; Rokala.</td>
</tr>
<tr>
<td>MI/TN</td>
<td>&quot;Naskapi&quot; has formal precedence</td>
</tr>
<tr>
<td>Naskapi</td>
<td>Also called &quot;Greek&quot; from Franglen (unpublished)</td>
</tr>
<tr>
<td>New Guinea</td>
<td>Slow</td>
</tr>
<tr>
<td>Oliphant</td>
<td>&quot;Slow&quot; Weikamp et al. may be same as &quot;Gombak&quot;</td>
</tr>
<tr>
<td>Ottsu</td>
<td>Same as &quot;Makiritare-1&quot;</td>
</tr>
<tr>
<td>Paris</td>
<td>Author refers to Geerdink (unpublished for source of sample)</td>
</tr>
<tr>
<td>Piaroa</td>
<td>Same as &quot;New Guinea&quot; and &quot;Maprik&quot;</td>
</tr>
<tr>
<td>Pollibauer</td>
<td>Author cites Porta et al. for source of sample</td>
</tr>
<tr>
<td>Pushtoon</td>
<td>Author cites Ortalli for source of sample</td>
</tr>
<tr>
<td>Reading</td>
<td>Author cites Ortalli for source of sample</td>
</tr>
<tr>
<td>Roma</td>
<td>Author cites Porta for source of sample</td>
</tr>
<tr>
<td>RS-I</td>
<td>Called &quot;Reading&quot; in Weikamp; &quot;Reading&quot; is formally precedent</td>
</tr>
<tr>
<td>RS-II</td>
<td></td>
</tr>
<tr>
<td>Santa Ana</td>
<td></td>
</tr>
<tr>
<td>Sentul</td>
<td></td>
</tr>
<tr>
<td>SO/BS</td>
<td></td>
</tr>
<tr>
<td>Syracuse</td>
<td></td>
</tr>
<tr>
<td>Tainsha</td>
<td></td>
</tr>
<tr>
<td>Welch &amp; Lie-Injo</td>
<td></td>
</tr>
<tr>
<td>Weikamp</td>
<td></td>
</tr>
<tr>
<td>Weikamp et al.</td>
<td></td>
</tr>
<tr>
<td>Izquierdo</td>
<td></td>
</tr>
<tr>
<td>Arends et al.</td>
<td></td>
</tr>
<tr>
<td>Geerdink et al.</td>
<td></td>
</tr>
<tr>
<td>Tanis et al.</td>
<td></td>
</tr>
<tr>
<td>Weikamp et al.</td>
<td></td>
</tr>
<tr>
<td>Weikamp &amp; Chagonon</td>
<td></td>
</tr>
<tr>
<td>McDermid</td>
<td></td>
</tr>
<tr>
<td>Lie-Injo et al.</td>
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<tr>
<td>Melartin &amp; Blumberg</td>
<td></td>
</tr>
<tr>
<td>Petrini et al.</td>
<td></td>
</tr>
<tr>
<td>Melartin &amp; Blumberg</td>
<td></td>
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<tr>
<td>Weikamp et al.</td>
<td></td>
</tr>
<tr>
<td>Weikamp et al.</td>
<td></td>
</tr>
<tr>
<td>Sandor et al.</td>
<td></td>
</tr>
<tr>
<td>Tanis et al.</td>
<td></td>
</tr>
<tr>
<td>Weikamp et al.</td>
<td></td>
</tr>
<tr>
<td>Weikamp &amp; Buck</td>
<td></td>
</tr>
<tr>
<td>Tarnoky &amp; Lestas</td>
<td></td>
</tr>
<tr>
<td>Weikamp et al.</td>
<td></td>
</tr>
<tr>
<td>Weikamp et al.</td>
<td></td>
</tr>
<tr>
<td>Kueppers et al.</td>
<td></td>
</tr>
<tr>
<td>Welch &amp; Lie-Injo</td>
<td></td>
</tr>
<tr>
<td>Weikamp et al.</td>
<td></td>
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<tr>
<td>Schneiderman et al.</td>
<td></td>
</tr>
<tr>
<td>Weikamp et al.</td>
<td></td>
</tr>
</tbody>
</table>

(continued overleaf)
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Table I (com.)

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acids carry no charge. In this case the variant molecules will behave identically in an electric field and could not be distinguished. It is possible, therefore, that albumin variants not detectable through electrophoresis will be discovered as the techniques for mapping the amino acid sequence of albumin, or of detecting non-charge amino acid differences, are more widely applied.

The first variants were discovered by paper electrophoresis and were characterized by electrophoretic mobilities markedly different from that of normal albumin. As new combinations of support medium, buffer and conductors were used in screening serum proteins new albumin variants were discovered. Many of these new variants have electrophoretic mobilities quite close to normal albumin and cannot be detected by paper or cellulose acetate electrophoretic systems used in routine screening but can be detected by the use of other support media (i.e. cellulose acetate or acrylamide gel; see, for example, Fig. 1). At present no single system is able to distinguish all the albumin variants from each other and from normal albumin.

A list of albumin variants that have appeared in the literature is presented here (Table 1). Some of these variants, however, have not yet been distinguished from other known variants by electrophoresis. It is possible that there are more names than there are actually different variants.

Recently Weitkamp et al. have compared samples of many of the variants using three starch gel systems. The first employs the sodium acetate-ethylenediaminetetraacetic acid system (pH 5.0) described by Kueppers and Bearn. (111)

Table 2. Relative mobility of twenty-five albumin variants in three starch gel electrophoretic systems (from Weitkamp et al.)

<table>
<thead>
<tr>
<th>Variant</th>
<th>Buffer system</th>
<th>pH 5.0</th>
<th>pH 6.0</th>
<th>pH 6.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. RS-I</td>
<td></td>
<td>1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>2. Pollibauer</td>
<td></td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3. Belem I</td>
<td></td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>4. B</td>
<td></td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>5. Roma</td>
<td></td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>6. Gainesville</td>
<td></td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>7. Paris (Gombak)</td>
<td></td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>8. Kashmir (Afghanistan)</td>
<td></td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>9. Otsu</td>
<td></td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>10. Santa Ana</td>
<td></td>
<td>7</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>11. SO/BS</td>
<td></td>
<td>7</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>12. Cartago</td>
<td></td>
<td>7</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>13. Xavante</td>
<td></td>
<td>8</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>14. Pushtoon</td>
<td></td>
<td>8</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>15. Cayemite</td>
<td></td>
<td>9</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>16. Mexico</td>
<td></td>
<td>9</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>17. Limba</td>
<td></td>
<td>9</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>18. Yanomama-2</td>
<td></td>
<td>10</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Normal albumin</td>
<td></td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>19. Medan</td>
<td></td>
<td>11</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>20. Maku</td>
<td></td>
<td>12</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>21. Reading (New Guinea)</td>
<td></td>
<td>13</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>22. Makiritare-3</td>
<td></td>
<td>13</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>23. Naskapi</td>
<td></td>
<td>14</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>24. Gent</td>
<td></td>
<td>15</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>25. Kyoto</td>
<td></td>
<td>16</td>
<td>11</td>
<td>10</td>
</tr>
</tbody>
</table>
Fig. 1. Electrophoretic patterns of serum albumin; vertical electrophoresis using a 5% acrylamide gel at pH 8.4. O = origin; + = direction of anode; (1) $AI^A/Al^{Na}$; (2) $Al^{Na}/Al^Na$; (3) $Al^{Na}/Al^{Na}$; (4) $AI^A/Al^A$. (From Johnston et al. (53)).
The second employs a tris-lithium-succinate-citrate gel buffer (pH 6.0) and a vessel buffer of the same composition but diluted 3:1. The third system has a tray buffer of 0.03 M tris (hydroxymethyl) amino-methane, 0.043 M ethylenediaminetetra-acetic acid, 0.27 M boric acid (pH 6.4) and a gel buffer of the same but diluted 1:6.2 (pH 6.9). With these three systems twenty-five variants were distinguished. These results are presented in Table 2 (adapted from Weitkamp).

Some variants (Table 2) which appear to have the same mobility in one electrophoretic system may be distinguished from each other by another combination of buffers and media. The most acidic buffer (pH 5.0) is best for comparing the fast variants but is relatively ineffective for distinguishing the slower ones. Both of the more alkaline systems are necessary to distinguish all of the slow variants. Screening for albumin variants with only one or two of these systems does not assure the investigator that all known variants will be detected.

Since Weitkamp's most recent comparison in 1973, there have been additional reports of albumin variants. Determining whether these variants are new or not requires the extensive comparisons just described. Since most investigators do not have a bank of variants for comparison, some of these "new" variants may be identical to those already discovered. These new variants include albumin Kuala Lumpur, albumin Birmingham, albumin Boal, albumin Luarca, and an unnamed variant discovered among Japanese emigres to Hawaii.

The recognition of these and future discoveries as "new" variants rests entirely upon comparisons with those variants already known. At present there is no centralized system for the extensive electrophoretic testing required to determine the status of "new" variants. Ultimately, the recognition of new species of albumin molecules will depend upon amino acid mapping, but until then the status of new variants will be somewhat ambiguous.

III. FAMILY STUDIES

A. Mode of Inheritance

When albumin variants were discovered in the families of propositi the hypothesis was advanced that the albumin phenotype was under genetic control. Since then numerous pedigrees have been published (examples are given in Fig. 2) all of which support the hypothesis that two autosomal codominant alleles, each having complete penetrance and segregating at one locus, control the albumin phenotype. Segregation analysis (Table 3) also supports this hypothesis.

B. Linkage to Other Loci

Pedigree data from families with alloalbuminemic members have also been analyzed to test the possibility of linkage between structural loci for albumin and other genetic systems. Table 4 presents a list of the genetic loci systems tested for linkage with the albumin locus. Several statistical techniques have been employed; the most common of these are those of Finney, Haldane and Smith, Morton and Smith.
Table 3. Segregation of albumin types in families (from Melartin (304))

<table>
<thead>
<tr>
<th>Albumin types of parents</th>
<th>No. of families</th>
<th>Albumin type of children</th>
<th>No. of children</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A/A</td>
<td>A/Na</td>
</tr>
<tr>
<td>A/A × Na/Na</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A/A × A/Na</td>
<td>14</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>A/A × A/Me</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Weitkamp et al. (31) and Kaarsalo et al. (81) initially recognized linkage between the albumin and Gc loci. Since these first analyses more pedigrees have become available in the literature. From a study of twenty-one pedigrees, fifteen from the literature and six new ones, Weitkamp et al. (31) concluded that the most likely value for the recombination fraction of albumin and the Gc genotypes is 0.023 (with 95% probability limits of 0.008 and 0.047). Furthermore, recombination values computed for each pedigree were about the same although six different albumin variants were segregating in the pedigrees (probably albumins B, Santa Ana, Gainesville, Syracuse, Naskapi and a dimeric variant). This suggests that all variants are allelic at the same locus. Bowen et al. (81) using the twenty-one pedigrees from Weitkamp et al. (31) and three new ones, computed a map distance for the albumin and Gc loci of 2.5 centimorgans with 95% probability limits of 0.9 to 5.0 centimorgans.
### Table 4. Linkage between albumin and other loci

<table>
<thead>
<tr>
<th>Genetic system</th>
<th>Linkage</th>
<th>Statistical methods (^{(*)})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group specific component (Gc)</td>
<td>+</td>
<td>?</td>
<td>Arends et al.(^{(43)})</td>
</tr>
<tr>
<td>+</td>
<td>a</td>
<td>Bowen et al.(^{(43)})</td>
<td>Kaarsalo et al.(^{(44)})</td>
</tr>
<tr>
<td>+</td>
<td>b, c, d, e</td>
<td>Kueppers et al.(^{(44)})</td>
<td>Sunderman et al.(^{(44)})</td>
</tr>
<tr>
<td>+</td>
<td>a, c, d, e</td>
<td>Weitkamp et al.(^{(47)})</td>
<td>Weitkamp et al.(^{(47)})</td>
</tr>
<tr>
<td>+</td>
<td>a, c, d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABO</td>
<td>-</td>
<td>a</td>
<td>Bowen et al.(^{(43)})</td>
</tr>
<tr>
<td>Rh</td>
<td>-</td>
<td>b, c, d, e</td>
<td>Kaarsalo et al.(^{(43)})</td>
</tr>
<tr>
<td>MNS</td>
<td>-</td>
<td>a</td>
<td>Kaarsalo et al.(^{(43)})</td>
</tr>
<tr>
<td>Duffy</td>
<td>-</td>
<td>b, c, d, e</td>
<td>Weitkamp et al.(^{(47)})</td>
</tr>
<tr>
<td>Kidd</td>
<td>-</td>
<td>a</td>
<td>Weitkamp et al.(^{(47)})</td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>b, c, d, e</td>
<td>Weitkamp et al.(^{(47)})</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>-</td>
<td>b, c, d, e</td>
<td>Weitkamp et al.(^{(47)})</td>
</tr>
<tr>
<td>Transferrin</td>
<td>-</td>
<td>b, c, d, e</td>
<td>Weitkamp et al.(^{(47)})</td>
</tr>
<tr>
<td>Methemoglobinemia</td>
<td>-</td>
<td>d</td>
<td>Scott and Wright(^{(44)})</td>
</tr>
<tr>
<td>Alpha chain haptoglobin</td>
<td>-</td>
<td></td>
<td>Kueppers et al.(^{(44)})</td>
</tr>
</tbody>
</table>

\(^{(*)}\) a, Haldane and Smith; \(^{(46)}\) b, Finney; \(^{(47)}\) c, Smith; \(^{(48)}\) d, Morton; \(^{(49)}\) e, Mohr.

There is a very low probability of linkage between the albumin locus and the other loci which have been tested. Scott and Wright\(^{(44)}\) reported that the probability of linkage between hereditary methemoglobinemia and albumin was less than 0.08. The probability of linkage between the albumin locus and the ABO, MNS, Duffy, Kidd, P, haptoglobin and transferrin loci is also small.\(^{(47,72,81,83)}\) Kueppers et al.\(^{(44)}\) have tentatively suggested that there may be linkage between the loci for albumin and the alpha chain of haptoglobin, but this hypothesis has not been tested by any other investigators.

### IV. THE DISTRIBUTION OF ALBUMIN VARIANTS

#### A. Classification

At present it is difficult to determine the exact number of albumin variants. There are approximately twenty-five variants which have been distinguished from each
other using one or more electrophoretic systems (see Table 2), but this figure represents less than half the number of albumin variants that have been reported. Undoubtedly the discrepancy is due to the use of several names to apply to a smaller number of variants. Several practices are responsible for the introduction of synonyms into the literature on albumin variation. Occasionally, a new variant is not named by its discoverer and is later given several names by investigators working independently. At other times a variant which had been given a provisional but formally precedent name is renamed. In some instances a variant which was thought to be new is later recognized to be identical with a previously described variant. The result of these practices is a proliferation of variant names, many of which are not commonly used or recognized.

A list of variants is presented in Table 1. This table includes variants which were not named by their discoverers, variants which have been renamed, variants which may be rediscoveries of previously identified variants and variants which appear in comparative studies but which have not been described in the literature individually. In Table 1 the variant name is listed (when given by the discoverer), notes on similarities to other variants, the geographical provenance (when given by the discoverer), the reference for the first mention of the variant, and when there are synonyms for a variant, the formally precedent name is indicated.

This extensive list serves several purposes. It serves as a reference for investigators interested in the original source of any albumin variant discovered. It may help to resolve the history of those variants which have been lost from the literature by multiple naming or by other means. Furthermore, it serves to document the history of the field, and is a testimony to the large amount of albumin variation and the difficulty of settling on a uniform system of reporting and naming albumin variants.

The variants listed in Table 1 can be classified according to their frequency and distribution. Variants may be either rare or polymorphic depending on whether they occur at a frequency of 1% or more. This distinction is based on E. B. Ford's classic definition of genetic polymorphism, "the occurrence together in the same habitat of two or more discontinuous forms or 'phases', of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation" (ref. 96, page 11). Although Ford's definition and his comments elsewhere (94) indicate that genetic polymorphisms are maintained by natural selection alone rather than by genetic drift or recurrent mutation, we cannot be certain that natural selection alone is responsible for the polymorphic albumin variants. The role of genetic drift, particularly drift that is caused by cultural practices and historical accident, cannot be accurately estimated in the American Indian populations where the few albumin variants that reach high frequencies occur. In the classification presented here, the term polymorphic means simply that the variant alleles occur at a frequency above 1%, and by implication at too high a frequency to be maintained by recurrent mutation.

The classes, rare and polymorphic, may be subdivided on the basis of distribution. There are three classes of rare variants: (1) unique variants which have been seen only in the family in which they were discovered; (2) restricted rare variants which occur in a small number of unrelated individuals of one ethnic group; (3) unrestricted rare variants which occur in a small number of unrelated individuals of different ethnic groups and presumably different genomes. The polymorphic variants may be
| Table 5. Classification of albumin variants by frequency and distribution (*) |
|-----------------------------|------------------------|------------------------|-----------------------------|
| Unique(b)                  | Restricted             | Unrestricted           | Polymorphic                |
| Belem I                     | B (Ann Arbor, Jensen  | Paris (Gombak)         | Warao (Makiritare,          |
| Birmingham                 | SO/CZ)                 | Reading (Maprik,       | Makiritare-I)              |
| Boal                        | Gainesville            | New Guinea,            | Yanomamo-2                 |
| Caracas                     | Gent (MI/FAST)         | Syracuse,              |                            |
| Carib                       | Kashmir (Afghanistan)  | Westcott               |                            |
| Cayemeite                   | Maku (Belem III,       |                        |                            |
| Luarca                      | Makiritare-2, Wapihana |                        |                            |
| Makiritare-3               | Medan                  |                        |                            |
| Pollihauer                  | Yanomamo               |                        |                            |
| Santa Ana                   |                        |                        |                            |
| Sentul                      |                        |                        |                            |
| Uinba                       |                        |                        |                            |

(*) The following variants from Table I cannot be classified since detailed information on the genetic relatedness or distribution of the individuals having the variants is not available: A2; Albuquerque; Alp; Kyoto; Larson; Oliphant; Otso; Pushoon; RS-I; RS-II; SO/BS; Xavante.

(b) As noted in the text, not all variants have been compared directly and so some may not be different.
either restricted to one ethnic group and locality, or dispersed among several ethnic
groups and localities. A classification of variants according to these criteria is
presented in Table 5.

B. Rare Variants

As can be seen from Table 5 most variants are unique. The number of unique
variants may be inflated somewhat since not all variants have been compared directly
to all others and so some may be identical to already described and named variants.

Of the restricted rare variants, albumins B, Gainesville and Gent have been found
only in families of European origin. Albumins Kuala Lumpur and Medan are re-
stricted to families of Far Eastern origin. Albumins Maku and Yanomama are found
only in South American Indian families. Although these variants are not unique they
are very rare.

Albumin Kashmir is restricted to families of Indo-Dravidian descent, but it occurs
more often than any of the other restricted rare variants. Data on albumin Kashmir
and other variants which also occur among Indo-Dravidian populations are presented
in Table 6. As can be seen from the table, several different variants appear to be
involved but this impression is misleading. Albumin Kashmir, originally described by

<table>
<thead>
<tr>
<th>Location and population</th>
<th>Variant name</th>
<th>Number tested</th>
<th>Number variant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>India</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Delhi</td>
<td>unnamed (Kashmir)</td>
<td>518</td>
<td>2</td>
<td>37</td>
</tr>
<tr>
<td>Madras</td>
<td></td>
<td>275</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>Bombay</td>
<td></td>
<td>485</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>Bombay (Parsis)</td>
<td></td>
<td>363</td>
<td>0</td>
<td>97</td>
</tr>
<tr>
<td>Bombay (Iranis)</td>
<td></td>
<td>33</td>
<td>0</td>
<td>97</td>
</tr>
<tr>
<td>West Bengal (Koaras)</td>
<td></td>
<td>202</td>
<td>0</td>
<td>98</td>
</tr>
<tr>
<td>Kerala (Kadars)</td>
<td></td>
<td>213</td>
<td>0</td>
<td>99</td>
</tr>
<tr>
<td>South Indians</td>
<td></td>
<td>50</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>England</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kashmir family (Pakistan)</td>
<td>Kashmir</td>
<td>?</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>Birmingham émigré (from India)</td>
<td>Birmingham screening</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afghanistan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gawargia (Pushtoon)</td>
<td>Afghanistan (Kashmir)</td>
<td>251</td>
<td>10</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Pushtoon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaysia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indians</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indians</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Africa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Albumins "Kashmir" and "Afghanistan" are electrophoretically identical. Albumin "Kashmir" is the formally precedent name.)
Tarnoky and Dowding,\(^{(65)}\) albumin Afghanistan \(^{(68)}\) and the unnamed variant reported by McDermid \(^{(31)}\) are indistinguishable when compared directly. \(^{(64, 65)}\) (McDermid's original suggestion of similarity between the unnamed Indian variant and albumins Paris and Gainesville may have been based on arithmetic descriptions of migration rates and not on direct comparisons.) Albumin Pushtoon is electrophoretically distinguishable from Kashmir \(^{(45)}\) and albumin Birmingham shows different binding properties, but not different electrophoretic mobility from albumin Kashmir. \(^{(29)}\) There are then three variants involved, Pushtoon, Birmingham and Kashmir. Weitkamp et al. \(^{(60, 61)}\) have suggested that albumin Kashmir may be a low-frequency polymorphism in Indo-Dravidian populations. This possibility is based on the presence of the variant in geographically diverse Indo-Dravidian populations from New Delhi (India), Natal (South Africa), Kashmir (Pakistan) and Gawargia (Afghanistan). The variant does not reach polymorphic frequencies, although calculation of the gene frequency is an estimate since alloalbuminemic individuals from one village may be related or may not have been ascertained through a random sampling of the population.

The unrestricted rare variants, albumins Paris and Reading, occur in both European and Asian populations. Albumin Paris was first discovered in a French family. \(^{(60)}\) Several years later a variant termed “Gombak” was discovered among Malays and Indians of Malaysia. \(^{(55)}\) In a direct comparison of the variants they displayed identical electrophoretic mobility, \(^{(60)}\) consequently the name “Paris” should be adopted to refer to this variant and the name “Gombak” dropped.

Similarly albumin Reading was first found in a European family \(^{(63)}\) and other variants, “Maprik” \(^{(67)}\) and “New Guinea” \(^{(45, 100)}\) from New Guinea, and “Syracuse” \(^{(66)}\) and “Westcott” \(^{(60)}\) from families of European origin, were subsequently found to be identical. \(^{(60)}\) Albumin Reading is the formally precedent name. Weitkamp et al. have suggested that the distribution of albumin Reading in New Guinea may not be accounted for solely by random genetic forces. \(^{(63)}\) The data from New Guinea on albumin Reading and albumin Uinba, a rare variant restricted to one village in New Guinea, are presented in Table 7. As can be seen from the table, albumin Reading just reaches the frequency of 1% which denotes a polymorphism when widely separated villages are combined to form one population.

C. Polymorphic Variants

Polymorphic variants, both restricted and dispersed ones, occur only among Native American populations. Furthermore, the dispersed polymorphic variants, albumins Naskapi and Mexico, have been detected only among North and Central American Indian and mestizo populations while the restricted polymorphic variants, albumins Yanomama-2 and Makiritare, are found only among South American Indian populations.

1. Restricted polymorphic variants

Two variants, albumins Yanomama-2 (Yan-2) and Makiritare, reach polymorphic frequencies within the ethnic groups for which they were named. Data on albumin variants in South America are presented in Table 8.
Table 7. Albumin variants from New Guinea populations

<table>
<thead>
<tr>
<th>Population and location</th>
<th>Variant name</th>
<th>Number tested</th>
<th>Number variant</th>
<th>Gene frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Guinea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highlanders</td>
<td></td>
<td>50</td>
<td>0</td>
<td>0.0</td>
<td>51</td>
</tr>
<tr>
<td>New Guinea natives</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Markham Valley</td>
<td>New Guinea (Reading) (^{(a)})</td>
<td>35</td>
<td>2</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Papondetta</td>
<td>&quot; &quot;</td>
<td>45</td>
<td>1</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Rossel Island</td>
<td>&quot; &quot;</td>
<td>31</td>
<td>4</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Other villages</td>
<td></td>
<td>613</td>
<td>0</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Total: relatives of Rossel Is. Proposit</td>
<td>New Guinea (Reading) (^{(a)})</td>
<td>724</td>
<td>7</td>
<td>0.001 (^{(b)})</td>
<td>40</td>
</tr>
<tr>
<td>Uniba Village</td>
<td>New Guinea (Reading) (^{(a)})</td>
<td>—</td>
<td>12</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Uniba</td>
<td>82</td>
<td>4</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>New Guinea natives</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maprik region</td>
<td>Maprik (Reading) (^{(a)})</td>
<td>595</td>
<td>1</td>
<td>0.002 (^{(b)})</td>
<td>41</td>
</tr>
<tr>
<td>New Guinea natives</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manus Islands</td>
<td></td>
<td>183</td>
<td>0</td>
<td>0.0</td>
<td>101</td>
</tr>
<tr>
<td>New Guinea natives</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eastern Highlands and Islands</td>
<td>New Guinea (Reading) (^{(a)})</td>
<td>1307</td>
<td>7</td>
<td>0.0053 (^{(b)})</td>
<td>81, McDermid (unpublished)</td>
</tr>
<tr>
<td>New Guinea natives Tuade speakers, Goilala</td>
<td></td>
<td>145</td>
<td>0</td>
<td>0.0</td>
<td>102</td>
</tr>
</tbody>
</table>

\(^{(a)}\) These gene frequencies were not published but were calculated for this paper from the published numbers of normal and heterozygotic individuals. They are estimates based on the application of the direct count method. In some cases individuals may be related.

\(^{(b)}\) Albumins "Reading", "New Guinea" and "Maprik" are electrophoretically identical. \(^{(2)}\) Albumin "New Guinea" is the formally precedent name.
Table 8. Albumin variants in South American populations

<table>
<thead>
<tr>
<th>Population and location</th>
<th>Variant name</th>
<th>Number tested</th>
<th>Number variant</th>
<th>Gene frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venezuela &amp; Brazil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yanomama</td>
<td>Yanomama-2 (tested)</td>
<td>2943</td>
<td>461 (30)**</td>
<td>0.076</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>(for Yan-2 only)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piaroa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Macushi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Wapishana</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Makiritare</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Xavante</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Total (Non-Yanomama)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Makiritare</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juduwaduna Village</td>
<td>—</td>
<td>71</td>
<td>0</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Santa Maris Village</td>
<td>—</td>
<td>154</td>
<td>0</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Wasana Village</td>
<td>Makiritare(-1)</td>
<td>42</td>
<td>3</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Chajorana Village</td>
<td></td>
<td>74</td>
<td>5</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Sharamana Village</td>
<td>—</td>
<td>47</td>
<td>0</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Belem Village</td>
<td>Makiritare(-1)</td>
<td>72</td>
<td>3</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Acanana Village</td>
<td></td>
<td>74</td>
<td>2</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Total</td>
<td>Makiritare(-1)</td>
<td>534</td>
<td>13</td>
<td>0.01</td>
<td>44</td>
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<tr>
<td>Warao</td>
<td>Warao (Makiritare)</td>
<td>222</td>
<td>5</td>
<td></td>
<td>43</td>
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<tr>
<td>Yanomama</td>
<td>Unnamed</td>
<td>984</td>
<td>49</td>
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<td>45</td>
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<tr>
<td>Makiritare</td>
<td></td>
<td>186</td>
<td>2</td>
<td></td>
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<tr>
<td>Piaroa</td>
<td></td>
<td>146</td>
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<td></td>
<td>45</td>
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<tr>
<td>Macushi</td>
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<td>45</td>
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<tr>
<td>Wapishana</td>
<td></td>
<td>62</td>
<td>15</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>Cayapo</td>
<td>—</td>
<td>89</td>
<td>0</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>Yanomama</td>
<td>Borabuk Village</td>
<td>Maku (Makiritare-2)</td>
<td>75</td>
<td>4</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Other villages</td>
<td>—</td>
<td>1036</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>Yanomama</td>
<td>Maku (Makiritare-2)</td>
<td>2256</td>
<td>4</td>
<td></td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Yanomama</td>
<td></td>
<td></td>
<td></td>
<td>103</td>
</tr>
<tr>
<td>Brazil</td>
<td>Belem (Trihybrid)</td>
<td>Belem I family</td>
<td>1360</td>
<td>5</td>
<td>46</td>
</tr>
<tr>
<td>-------------</td>
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<td>------------------</td>
<td>------</td>
<td>---</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>Belem II (Mexico)</td>
<td>&quot;</td>
<td>&quot;</td>
<td>6</td>
<td>46</td>
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<tr>
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<td>Belem III</td>
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<td>46</td>
</tr>
<tr>
<td>Porto Alegre (Trihybrid)</td>
<td>—</td>
<td>1628</td>
<td>0</td>
<td>46</td>
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<tr>
<td>Xavante</td>
<td>San Marcos Village</td>
<td>Xavante</td>
<td>214</td>
<td>2</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Sao Diminos and</td>
<td>&quot;</td>
<td>&quot;</td>
<td>245</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Sinoes Lopes villages</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
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<tr>
<td>Cayapo</td>
<td>Xikrin Village</td>
<td>&quot;</td>
<td>95</td>
<td>0</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Grorotire Village</td>
<td>&quot;</td>
<td>201</td>
<td>0</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Kuben-kran-kegn Village</td>
<td>&quot;</td>
<td>164</td>
<td>0</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Mekranoti Village</td>
<td>&quot;</td>
<td>92</td>
<td>0</td>
<td>104</td>
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<tr>
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<td>Txukahamae Village</td>
<td>&quot;</td>
<td>156</td>
<td>0</td>
<td>104</td>
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<tr>
<td>Surinam</td>
<td>Trio</td>
<td>Makiritare-1</td>
<td>158</td>
<td>9</td>
<td>47</td>
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<tr>
<td></td>
<td>Pelelu Tepu Village</td>
<td>&quot;</td>
<td>255</td>
<td>2</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Alalaparu Village</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>Wajana</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>Puleowime Village</td>
<td>Makiritare-1</td>
<td>81</td>
<td>7</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Kawemkakan Village</td>
<td>&quot;</td>
<td>198</td>
<td>10</td>
<td>47</td>
</tr>
<tr>
<td>Colombia</td>
<td>Noanama</td>
<td>&quot;</td>
<td>155</td>
<td>0</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Cofan</td>
<td>&quot;</td>
<td>68</td>
<td>0</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Ingana (Quechua)</td>
<td>&quot;</td>
<td>41</td>
<td>0</td>
<td>105</td>
</tr>
<tr>
<td>Peru</td>
<td>Quechua</td>
<td>&quot;</td>
<td>100</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Cashinahua</td>
<td>&quot;</td>
<td>92</td>
<td>0</td>
<td>30</td>
</tr>
</tbody>
</table>

Figure in parentheses refers to number of homozygotes.
**Yanomama-2 is found at a frequency of 7.6% among sixty-four Yanomama villages from southern Venezuela and northern Brazil.** The frequency tends to be highest in the northern area; the highest value for any village is 40%. Screening specifically for Yanomama-2, which requires a novel electrophoretic system, has been performed on 2562 sera from several tribes of North and South America. In only one case, and that from the nearby Makiritare tribe, was Yanomama-2 detected. Hence, Yanomama-2 appears to be polymorphic within a single ethnically defined group.

Albumin Makiritare (also called Warao) has been found among the Trio and Wajana tribes of Surinam, the Warao of Venezuela and the Makiritare. It reaches a frequency of 4% in some Makiritare villages and may be even more frequent in some Wajana villages. The average frequency for the variant in seven Makiritare villages is 1%. Albumin Makiritare reaches polymorphic frequencies among several tribal groups, but its distribution appears to be limited to the northeastern area of South America only.

**2. Dispersed polymorphic variants**

(a) Distribution. The only variants that have been found at polymorphic frequencies in samples from several widely separated geographic areas are albumins Naskapi and Mexico. These variants are found only among American Indian populations where they often reach frequencies above 1%. The distribution of these variants reaches from Alaska to Guatemala. Albumin Naskapi is found predominantly among northern populations while albumin Mexico is found among Middle American populations. Both variants are found among some populations in the greater southwest. In this survey the populations are grouped by linguistic affiliation to facilitate description. In the subsequent sections linguistic groupings form only a part of the interpretation.

Table 9 shows the distribution of albumin variants among Native Americans of North and Middle America. (Stated in this table are the linguistic affiliation, tribal name, location, total number in the sample, number of albumin Naskapi heterozygotes and, in parentheses, homozygotes, the number of albumin Mexico heterozygotes, and homozygotes in parentheses, and the gene frequencies of each variant. Gene frequencies are direct count estimations from the frequency of heterozygotes and homozygotes. Some authors have calculated gene frequencies with a correction factor for including related individuals in the populations; these data are noted in parentheses.)

Albumin Naskapi is found at high frequencies among the Algonkian-speaking Naskapi and Montagnais Indians of Quebec, among Athabascan-speaking Slave and Beaver Indians of Alberta and among Athabascan Indians of Alaska. Lower frequencies, from 0.5% to 3.4%, are found among the Sioux, Assiniboine, Ojibwa, Cree, Chippewa and Blackfoot who are found in the plains and forested regions of the United States and Canada.

Very low frequencies of albumin Naskapi had been reported for a Tlingit Indian sample and for two Eskimo samples. Some of the alloalbuminemic individuals within each sample were later found to be of mixed ancestry, or of a different ethnic group than had originally been supposed. (All the samples in question were collected...
near Indian populations where the Naskapi gene is present.) Albumin Naskapi now appears to be absent from the Tlingit and Haida Indians of the Canadian Pacific coast and from numerous Eskimo samples from Alaska to Greenland. Albumin Naskapi is also absent from samples of Omaha Indians of the Macy reservation and from Floridian Indians. Albumin Mexico has not been found in any of the northern Native Americans who have been tested.

The distribution of albumin Mexico is limited to more southern regions. Among Middle American Indian and Mestizo populations albumin Mexico is occasionally found at polymorphic frequencies. It is present from Tamulipas, Mexico, on the Gulf Coast in the north to Guatemala City, Guatemala, in the south. The highest frequencies of albumin Mexico in Middle America have been reported for two Mestizo samples, both in Tlaxcala and Hidalgo, Mexico.

Albumin Mexico also reaches polymorphic frequencies in the southwest United States. The highest frequencies have been found among the Yuman River-speaking Maricopa and Cocopah and among the Uto-Aztecan-speaking Pima and Zuni. Lower frequencies have been noted for Papago samples. Several other samples from southwest tribes have been tested, but these samples are too small for an accurate estimate of gene frequency.

Occasionally, both albumin Mexico and Naskapi are found in the same population. These occurrences are limited to several southwestern groups, the Navajo and Apache and the Mohave and Maricopa. Within each group, the proportion of the variants to each other and to common albumin is different (see Table 9).

(b) Anthropological interpretation. These differences suggest that albumin variants may be a useful indicator of contacts and movements among Native American populations. There are features of the distribution of albumin variants which support anthropologists' theories regarding relationships among Native American peoples.

Johnston et al. noted that the distribution of albumin Mexico in Guatemala paralleled the archaeological evidence of past cultural relationships there. Archaeological evidence suggests that the cultures of the Guatemalan highlands were greatly influenced by a northern culture, perhaps one centered near the Valley of Mexico which surrounds the modern Mexico City. The distribution of albumin Mexico reflects this relationship, being present in the Guatemalan highlands but absent from the lowland Yucatan where influence from the Valley of Mexico was presumably less intense.

The distribution of albumin Mexico to the north of the Valley of Mexico may parallel cultural relationships there also. Archaeological research suggests that there may have been contact between the Valley of Mexico cultures and peoples in the southwest United States, in particular, the Hohokam culture and peoples from the area around Tamulipas, Mexico. Albumin Mexico has been found at polymorphic frequencies in a sample from Tamulipas, in Pima samples and in one Papago sample. The Pima and Papago are thought to be descendants of the Hohokam. The presence of albumin Mexico among them may reflect earlier contact with Valley of Mexico peoples, and if so, the far higher frequency of albumin Mexico among the Pima may further indicate much more contact with the Pima than with the Papago. The distribution of albumin Mexico appears to parallel the area of Valley of Mexico influence in the American southwest as well as in Guatemala although the direction of gene flow and cultural contact cannot be presumed from distributional data.
<table>
<thead>
<tr>
<th>Linguistic affiliation, tribal name (location)</th>
<th>Number tested</th>
<th>Number Naskapi (homozygotes in parentheses)</th>
<th>Number Mexico (homozygotes in parentheses)</th>
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<td>1</td>
<td></td>
<td></td>
<td>111</td>
</tr>
<tr>
<td>Mestizo-Mixtec</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(San Pedro Mixtec, Mexico)</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td>111</td>
</tr>
<tr>
<td>(Ometepec, Mexico)</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td>111</td>
</tr>
<tr>
<td>(Cuajinquisquila, Mexico)</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td>111</td>
</tr>
<tr>
<td>Mestizo (Tlaxcala, Hidalgo &amp; Mexico, D.F., Mexico)</td>
<td>185</td>
<td>0</td>
<td>10</td>
<td>0.0</td>
<td>0.027</td>
<td>111</td>
</tr>
<tr>
<td>Mestizo (Tlaxcala–Hidalgo, Mexico)</td>
<td>257</td>
<td>0</td>
<td>12</td>
<td>0.0</td>
<td>0.023</td>
<td>106</td>
</tr>
<tr>
<td>Mestizo (Veracruz, Mexico)</td>
<td>109</td>
<td>0</td>
<td>1</td>
<td>0.0</td>
<td>0.004</td>
<td>106</td>
</tr>
<tr>
<td>Mestizo (Campeche, Mexico)</td>
<td>109</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>106</td>
</tr>
<tr>
<td>Mestizo (West Coast, Mexico)</td>
<td>99</td>
<td>0</td>
<td>1</td>
<td>0.0</td>
<td>0.005</td>
<td>106</td>
</tr>
<tr>
<td>Mestizo (Mexico City, Mexico)</td>
<td>1313</td>
<td>0</td>
<td>27</td>
<td>0.0</td>
<td>0.010</td>
<td>106</td>
</tr>
</tbody>
</table>

*Published gene frequency, calculated with correction for related individuals.*

*Very low frequency was reported originally; this value has since been corrected to the value shown here.*
The distribution of albumin Naskapi also parallels some cultural relationships among Indian groups of the American southwest. The Navajo and Apache for whom polymorphic frequencies of albumin Naskapi have already been noted are closely related linguistically to Canadian and Alaskan Athabascan-speaking Indians. Archaeological research suggests that the Navajo and Apache gradually moved into the southwest from 500 to 1000 years ago. Glottochronological analyses support these dates. The presence of albumin Naskapi among them may therefore be due to their past relationship with northern peoples who maintain albumin Naskapi at polymorphic frequencies. The presence of albumin Naskapi among indigenous peoples of the southwest may be due to contact with the Apache and Navajo. Indeed, there is evidence for such contact within the last 300 years.

The distribution of albumin Naskapi in the far north substantiates theories regarding the relationship between Eskimos and Indians. Although both groups are considered Native Americans, a distinction between them has been made on biologic and cultural grounds (see, for example, references 117, 122 and 123). The absence of albumin Naskapi from most Eskimo samples and its presence among Indians lends support to this distinction.

Although the distribution of albumin Naskapi may reflect movements and contacts among Native Americans, the presence of the gene at polymorphic frequencies in some areas may have been produced by other factors. The nature of these evolutionary forces is not known, nor is it likely to be detected solely from distributional studies. However, the distribution of the gene might indicate environments where albumin Naskapi has been maintained at polymorphic frequencies by natural selection. For example, research into the functional differences between albumins, particularly into the different drug-binding properties of albumin Naskapi and common albumin (A1/A1), might consider their activity with regard to substances common to the ecologies of the Naskapi, Montagnais, Slave, Beaver and Alaskan Indians. If selective forces operate on the albumin locus, they may be more apparent among those groups where albumin Naskapi is more frequent.

V. SUMMARY

The human serum albumin phenotype is controlled by two autosomal codominant alleles which segregate at a single locus. The albumin and Group Specific component loci are closely linked.

Variants of human serum albumin are indistinguishable from normal albumin by immunologic and ultracentrifugation techniques. The variants are distinguishable by their electrophoretic mobilities. There are approximately twenty-five electrophoretically distinguishable variants.

Most of these variants are unique although a few variants from different families have identical electrophoretic mobilities. Other variants, such as "Kashmir" and "New Guinea", may be more common, but do not reach a frequency of 1%, and so may not be polymorphic. Albumin polymorphisms are known from Native American populations only. In South America there are two variants, albumin Makiritare and Yanomama-2, which reach polymorphic frequencies in limited areas. In North America albumin Naskapi and Mexico reach polymorphic frequencies in several
widely separated geographic regions. The distribution of albumin Naskapi and Mexico may parallel relationships among Native American peoples and reflect their past movements and contacts. The evolutionary forces responsible for the high frequency of albumin variants in some areas are not known.

REFERENCES


ADDENDUM

Chapter 5

Blood Polymorphisms and the Origins of New World Populations

Michelle Lampl and Baruch S. Blumberg

Introduction

An objective of this volume is to discuss methods that may be used to identify contemporary Asian populations that have affinities with contemporary native American populations by virtue of their descent from the same ancestral population. If such populations exist, their similarity to American populations can be demonstrated if they have retained genes present in the ancestral populations and in their related populations in the Americas. These would be particularly useful if they included genes of polymorphic loci found only in native Americans and their Asian counterparts. In this chapter we will describe genes that may be useful in such comparisons. It is obvious that if gene frequencies have altered very differently in the two continents by virtue of drift, gene admixture, or differences in selection in the two environments, then a comparison using this technique would not be possible. Hence, this discussion is based on the assumption that it is possible to identify similarities between North American and Asian populations by comparisons using the appropriate genes.

There are two systems (serum albumins and white blood cell HL-A antigens) for which a relatively large amount of data is available and these are discussed in greatest detail. In addition, other systems that may be useful are mentioned.

Albumin Variants

The albumin polymorphisms may be one of the most useful markers for the identification of American Indian affinities. Two genes of relatively wide distribution, albumin Naskapi and albumin Mexico, have been identified in North
and Central American Indian populations. Two other genes of relatively restricted distribution, albumin Makiritare and albumin Yanomama-2, have been found in South American populations. Although rare variants (i.e., fewer than one per 100) have been found in European, Asian, and Oceanic populations (i.e., albumin B, albumin Kashmir), in no case have they reached the 1 percent figure characteristic of genetic polymorphisms. Hence, if a polymorphism for albumin is identified in an Asian population whose affinities with American Indians is suspected, and in particular if the polymorphic variant has the same electrophoretic mobility and other characteristics as Naskapi, Mexico, Makiritare, or Yanomama, then this would provide a strong argument in favor of the association of that population with American Indians (Schell and Blumberg 1977).

Human serum albumin phenotypes are determined by autosomal codominant alleles which segregate at a single locus. Albumin variants are distinguished by their electrophoretic mobilities (often using several techniques for the separations) and approximately 25 such variants have so far been identified (Table 5.1). Most of these variants occur rarely (one in several thousand) and presumably are maintained in the population primarily as a consequence of recurrent mutation.

Table 5.1. Relative Mobilities of 25 Albumin Variants in Three Starch Gel Electrophoretic Systems*

<table>
<thead>
<tr>
<th>Variant</th>
<th>Buffer system</th>
<th>Buffer system</th>
<th>Buffer system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5.0</td>
<td>pH 6.0</td>
<td>pH 6.9</td>
</tr>
<tr>
<td>1. RS-1</td>
<td>1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>2. Polibauer</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3. Belem 1</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>4. B</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>5. Roma</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>6. Gainesville</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>7. Paris (Gombak)</td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>8. Kashmir (Afghanistan)</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>9. Otsu</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>10. Santa Ana</td>
<td>7</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>11. SO/BS</td>
<td>7</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>12. Cartago</td>
<td>7</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>13. Xavante</td>
<td>8</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>14. Pushutan</td>
<td>8</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>15. Cayemite</td>
<td>9</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>16. Mexico</td>
<td>9</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>17. Uniba</td>
<td>9</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>18. Yanomama-2</td>
<td>10</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Normal albumin</td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>19. Medan</td>
<td>11</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>20. Maku</td>
<td>12</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>21. Reeling (New Guinea)</td>
<td>13</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>22. Makiritare-5</td>
<td>13</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>23. Naskapi</td>
<td>14</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>24. Gent</td>
<td>15</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>25. Kyoto</td>
<td>16</td>
<td>11</td>
<td>10</td>
</tr>
</tbody>
</table>

* From Weitkamp (1973).
The present chapter considers the four variants that reach polymorphic frequencies: Two of these have been observed in widely separated geographic regions (Naskapi and Mexico), whereas the other two have been primarily confined to the populations in which they were first discovered (Makiritare and Yanomama).

The first albumin variant to be found at polymorphic frequency was reported by Melartin and Blumberg (1965). It has a mobility faster than the common albumin A. It was found originally in the relatively isolated Naskapi and Montagnais Indians of Quebec Province, Canada and was termed albumin Naskapi. This study also contained the first report of individuals homozygous for an albumin other than the common albumin A. In 1967, a slow moving variant (albumin Mexico) was found in polymorphic frequency among Indians of the American Southwest and Mexico (Melartin et al. 1967). These variants (Naskapi and Mexico) have not been reported in populations other than American Indians, even though many tens of thousands of sera have been examined by the appropriate electrophoretic methods.

Albumin Naskapi is found predominantly among northern populations, whereas albumin Mexico is found among southeastern United States and middle American populations. Populations in the southwest United States have both Naskapi and Mexico (Table 5.2, Figure 5.1). Table 5.2 is adopted from Schell and Blumberg (1977) and Figure 5.1 is taken from Schell et al. (1978).

The highest frequency of the albumin Naskapi gene (13 percent) is found in the eastern regions of North America, among the Algonkian-speaking Naskapi of Schefferville, Quebec. High gene frequencies (8 percent) are also found among the Montagnais Indians from the same region. Naskapi living at Northwest River, however, have a lower frequency of $A^{\text{Naskapi}}$ (3 percent). Albumin Naskapi was not found in samples of Mohawk and Micmac Indians from the St. Lawrence River area, and this river may represent the southern limit of the distribution of this variant in the east (Schell et al. 1978).

In the Ungava region of northern Quebec a few cases of albumin Naskapi have been found among Eskimos living close to Indian groups with a high frequency of the Naskapi variant. These appear to result from admixture, as no variant has been found among the more remote Eskimo populations from Alaska, Canada, and Greenland (McAlpine et al. 1974, Melartin 1967, Melartin and Blumberg 1966, Persson et al. 1971). Albumin Naskapi is also apparently absent from the Haida and Tlingit Indians of the Canada Pacific coast (Melartin 1967, Melartin and Blumberg 1966).

Albumin Naskapi, however, is found at high gene frequencies among Athabascan Indians of Alaska (2.8–5.1 percent) (Melartin and Blumberg 1966, Scott and Wright 1969) and Athabascan-speaking Slave and Beaver Indians of Canada (9.8 percent) (Bowen et al. 1971). Lower frequencies (less than 3.4 percent) are found among a number of other central and western Indian groups; Sioux (Melartin and Blumberg 1966), Assiniboine (Bowen et al. 1971), Ojibwa (Polasky and Rokala 1967, Szathmary et al. 1974, Tanis et al. 1974), Cree (Bowen et al. 1971), Chippewa (Tanis et al. 1974, Weitkamp 1973, Weitkamp et al. 1968), and
Table 5.2. The Distributions of Albumin Mexico and Albumin Naskapi in North and Central America

<table>
<thead>
<tr>
<th>Linguistic affiliation, Tribal name (location)</th>
<th>Number tested</th>
<th>Number Naskapi</th>
<th>Number Mexico</th>
<th>Naskapi gene frequency</th>
<th>Mexico gene frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algonkian</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ojibwa (No. Ontario)</td>
<td>117</td>
<td>6</td>
<td>0</td>
<td>0.026</td>
<td>0.0</td>
<td>Szathmary et al. (1974)</td>
</tr>
<tr>
<td>Ojibwa (So. Ontario)</td>
<td>94</td>
<td>1</td>
<td>0</td>
<td>0.005</td>
<td>0.0</td>
<td>Szathmary et al. (1974)</td>
</tr>
<tr>
<td>Ojibwa (?)</td>
<td>120</td>
<td>8</td>
<td>0</td>
<td>0.033</td>
<td>0.0</td>
<td>Tanis et al. (1974)</td>
</tr>
<tr>
<td>Ojibwa/Anglo (No. Minnesota)</td>
<td>250</td>
<td>11 + (1)</td>
<td>0</td>
<td>0.026</td>
<td>0.0</td>
<td>Polesky and Rohala (1967)</td>
</tr>
<tr>
<td>Chippewa/Cree (Saskatchewan)</td>
<td>610</td>
<td>36 + (1)</td>
<td>0</td>
<td>0.031</td>
<td>0.0</td>
<td>Hettig (1973)</td>
</tr>
<tr>
<td>Chippewa/Cree (Saskatchewan)</td>
<td>102</td>
<td>2</td>
<td>0</td>
<td>0.009</td>
<td>0.0</td>
<td>Hettig et al. (1968)</td>
</tr>
<tr>
<td>Chippewa (?)</td>
<td>87</td>
<td>2</td>
<td>0</td>
<td>0.011</td>
<td>0.0</td>
<td>Tanis et al. (1974)</td>
</tr>
<tr>
<td>Cree, Plais (Alberta)</td>
<td>605</td>
<td>25 + (1)</td>
<td>0</td>
<td>0.022 (0.023)</td>
<td>0.0</td>
<td>Bowen et al. (1971)</td>
</tr>
<tr>
<td>Cree, Northern (Alberta)</td>
<td>187</td>
<td>10</td>
<td>0</td>
<td>0.026 (0.034)</td>
<td>0.0</td>
<td>Bowen et al. (1971)</td>
</tr>
<tr>
<td>Blackfoot (Montana)</td>
<td>97</td>
<td>4</td>
<td>0</td>
<td>0.020</td>
<td>0.0</td>
<td>Polesky and Rekala (1967)</td>
</tr>
<tr>
<td>Naskapi (Schefferville, Quebec)</td>
<td>151</td>
<td>37 + (1)</td>
<td>0</td>
<td>0.150</td>
<td>0.0</td>
<td>Melanin and Blumberg (1966)</td>
</tr>
<tr>
<td>Montagnais (Schefferville, Quebec)</td>
<td>112</td>
<td>3 + (2)</td>
<td>0</td>
<td>0.080</td>
<td>0.0</td>
<td>Melanin and Blumberg (1966)</td>
</tr>
<tr>
<td>Naskapi (N.W. River, Labrador)</td>
<td>66</td>
<td>2</td>
<td>0</td>
<td>0.03</td>
<td>0.0</td>
<td>Schell et al. (1978)</td>
</tr>
<tr>
<td>Micmac (Restigouche, Quebec)</td>
<td>102</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>Schell et al. (1978)</td>
</tr>
<tr>
<td>Iroquois</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mohawk (Caugh Nawaga, Quebec)</td>
<td>112</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>Schell et al. (1978)</td>
</tr>
<tr>
<td>Siouan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sioux (?)</td>
<td>160</td>
<td>2</td>
<td>0</td>
<td>0.007</td>
<td>0.0</td>
<td>Melanin and Blumberg (1966)</td>
</tr>
<tr>
<td>Assiniboine (Alberta)</td>
<td>100</td>
<td>1</td>
<td>0</td>
<td>0.005 (0.007)</td>
<td>0.0</td>
<td>Bowen et al. (1971)</td>
</tr>
<tr>
<td>Omaha (Macy Reservation, Nebraska)</td>
<td>56</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>Schell et al. (1978)</td>
</tr>
<tr>
<td>Eskimo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eskimo (Igloolik, Canada)</td>
<td>356</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>McAlpine et al. (1974)</td>
</tr>
<tr>
<td>Eskimo (E. Greenland)</td>
<td>78</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>Person et al. (1971)</td>
</tr>
<tr>
<td>Eskimo (Thule, Greenland)</td>
<td>297</td>
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<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>Person et al. (1971)</td>
</tr>
<tr>
<td>Eskimo (W. Greenland)</td>
<td>116</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>Person et al. (1971)</td>
</tr>
<tr>
<td>Eskimo (Wainwright, Alaska)</td>
<td>111</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>Melanin and Blumberg (1966)</td>
</tr>
<tr>
<td>Eskimo (Anaktuvuk)</td>
<td>55</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>Melanin and Blumberg (1966)</td>
</tr>
<tr>
<td>Eskimo (Ungava Bay, Canada)</td>
<td>124</td>
<td>3</td>
<td>0</td>
<td>0.012</td>
<td>0.0</td>
<td>Melanin et al. (1968)</td>
</tr>
<tr>
<td>Eskimo (Barrow, Alaska)</td>
<td>88</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>Melanin et al. (1968)</td>
</tr>
<tr>
<td>Eskimo (Beaver, Alaska)</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>Melanin et al. (1968)</td>
</tr>
<tr>
<td>Population</td>
<td>M/M</td>
<td>M/FA</td>
<td>FA/FA</td>
<td>Cytogenetic</td>
<td>Melanin</td>
<td></td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-----</td>
<td>------</td>
<td>-------</td>
<td>--------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Eskimo (St. Lawrence Is., Alaska)</td>
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<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Eskimo (Nat'l. Guard, Alaska)</td>
<td>107</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Aleut (Sitka and Nat'l. Guard, Alaska)</td>
<td>15</td>
<td>0*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Eskimo (Frobisher Bay Ballin Island N.W. Terr.)</td>
<td>101</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Na-Déné (non-Athabascan branch)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thngit (Sitka and Mt. Edgecomb, Alaska)</td>
<td>91</td>
<td>0*</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Haida (Queen Charlotte Is., British Columbia)</td>
<td>365</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Na-Déné (Athabascan branch)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alaskan Athabascans (Alaska)</td>
<td>230</td>
<td>11 + (1)</td>
<td>0</td>
<td>0.028</td>
<td>0.0</td>
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<td>0.090 (0.098)*</td>
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<tr>
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**Table Continued**
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<tr>
<th>Linguistic affiliation, Tribal name (location)</th>
<th>Number tested</th>
<th>Number Naskapi*</th>
<th>Number Mexico*</th>
<th>Naskapi gene frequency</th>
<th>Mexico gene frequency</th>
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<td>Lisker et al. (1971)</td>
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<td>Lisker et al. (1971)</td>
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<tr>
<td>Mazatec (Huatula de Jimenez, Mexico)</td>
<td>20</td>
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<td>0</td>
<td>0.0</td>
<td></td>
<td>McLaren et al. (1967)</td>
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<tr>
<td>Mixe (Oaxaca, Mexico)</td>
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<td>Lisker et al. (1971)</td>
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<td>McLaren et al. (1967)</td>
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<td>McLaren et al. (1967)</td>
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<td>0</td>
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<td>McLaren et al. (1967)</td>
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<tr>
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<tr>
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<td>27</td>
<td>0.0</td>
<td>0.010</td>
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</tbody>
</table>

* Adapted from Schell and Blumberg (1977).
* Homozygotes in parentheses.
* Published gene frequency, calculated with correction for related individuals.
* A very low frequency was reported originally; this value has since been corrected to the value shown here.
Blackfoot (Polesky and Rokala 1967), although the variant is absent in others, i.e., Omaha (Schell et al. 1978). It also occurs in relatively high frequency in the Athabascan-speaking Navajo and Apache (Schell et al. 1978).

This wide geographic dispersion is consistent with some linguistic and cultural-historical data. From archaeologic evidence it is postulated that the movement of the Navajo and Apache into the Southwest from the North took place about 500-1000 years ago (Aikens 1970). The Navajo and Apache, furthermore, are linguistically related to Canadian and Alaskan Athabascan-speaking Indians, and glottochronologic analyses support these dates (Hoijer 1956, Hymes 1957).

Figure 5.1. Distribution of albumins Naskapi and Mexico in native American populations. (From Schell et al. 1978.)
In the southern regions of North America and in Central America the slow albumin variant, albumin Mexico, has been found in a distribution extending from the southwest United States to Guatemala. Albumin Mexico has not (at this time) been found in any other native northern North American population. Polymorphic frequencies of albumin Mexico have been found among the Uto-Aztecan-speaking Pima (Johnston et al. 1969, Poletsky et al. 1968) and Papago (Brown and Johnson 1970, Johnston et al. 1969, and Melartin 1967), the Zuni (Brown and Johnson 1970), and the Yuman River-speaking Maricopa and Cocopah (Weitkamp 1973).

The highest frequencies of albumin Mexico in middle America are reported for two Mestizo samples (Lisker et al. 1971, Melartin et al. 1967) and the variant is present at lower frequencies in populations from Tamulipas, Mexico on the Gulf coast (Lisker et al. 1971) to Guatemala City, Guatemala (Johnston et al. 1973).

The distribution of albumin Mexico parallels archeologic data for past cultural contacts (Weaver 1972, and Wormington 1970). The evidence suggests that a culture centered near the Valley of Mexico (area of modern Mexico City) had contacts with the peoples of the Guatemalan highlands, to the north with the Hohokam culture in southwestern United States and with people from the area of Tamulipas, Mexico. The Pima and Papago are thought to be descendants of the Hohokam (Spencer and Jennings 1965).

Among several southwestern United States groups (i.e., Navajo, Apache, Mohave, and Maricopa), both albumins Mexico and Naskapi are found concurrently at varying frequencies (Johnston et al. 1969).

It is interesting that albumin Mexico is found among the Navajo and, to a greater extent, among the Apache, but albumin Naskapi is only rarely found among the Uto-Aztecan tribes. This is consistent with ethnohistorical evidence that, as a result of warfare and kidnapping, Pima and Papago and others were assimilated into the Navajo and Apache groups but the reverse happened less often.

In South America the two geographically restricted variants (albumins Yanomama-2 and Makiritare) are found in polymorphic frequencies in the ethnic groups after which they are named and in some nearby groups. Yanomama-2 has been found at a frequency of 7.6 percent among 64 Yanomama villages in southern Venezuela and Northern Brazil. The highest frequency of Yanomama-2 (40 percent) has been found in a northern village (Tanis et al. 1974).

Albumin Makiritare (which is identical with albumin Warao) has been found among the Makiritare, the Warao of Venezuela, and the Trio and Wajana tribes of Surinam (Arends et al. 1969, 1970, Geerdink et al. 1971). The variant reaches polymorphic frequencies among several tribal groups, but its distribution is confined to the northeastern area of South America. Although the average frequency for the albumin in the Makiritare is about 1 percent it reaches a frequency of 4 percent in several individual villages.

Hence, polymorphic variants of albumin have been found only in American Indians (but not Eskimos). The identification of similar variants in Asian populations could contribute to our understanding of affinities with Native Americans.
The HL-A System (Human Major Histocompatibility Complex)

The HL-A system is thought to be the major human histocompatibility system (apart from ABO) in humans and is present as an antigenic polymorphism on many human tissues. The HL-A antigens were first found as isooagglutinogens on platelets and white blood cells, and routine determinations utilize these cells. Subsequent research has revealed their medical importance in determining whether and to what extent the tissues of a potential transplant donor are compatible with the putative recipient. Histocompatibility antigens show a high degree of polymorphism, and the great variation of antigen frequencies in different populations makes the HL-A system a potentially powerful genetic marker for population studies. Research into the histocompatibility system is complicated by the large number of antigenic specificities and the difficulties in standardizing reagents. Despite this, these systems may prove to be among the most important genetic systems for studying relations between human populations. We use it here as an example of how such data may be applied to the study of Asian populations that may be related to Americans. A revised system of nomenclature for the HL-A system has recently been introduced (Amos et al. 1976). To maintain consistency with the published population data the earlier nomenclature is used here.

It is now generally assumed that the HL-A system consists of four autosomal linked loci, now termed A, B, C, and D, each with a complex series of allelic genes giving rise to distinct antigens. (The actual sequence on the chromosome is D, B, C, A.) Of these, the antigens defined by the A, B, and C loci are serologically determined and the D locus antigens are responsible for the mixed lymphocyte response (MLR locus). An HL-A haplotype generally refers to a section of a chromosome containing the A (or LA) locus and the B (second or fourth locus) (i.e., a pair of antigens). The reagents for the C locus were not generally available until recently. The use of haplotype designations, as we shall see later, can lead to very specific identifications of populations. There are some population-related antigens at each of the loci. HL-A1 is characteristic of Caucasians, whereas HL-A3 and HL-A8 characterize non-Caucasian populations. HL-A9 is high among Orientals, New Guineans, New Zealand Maoris, Eskimos, and American Indians. W10 is high among populations of Asians and North and South American Indians; and W15 is commonly found in Asia and South America (Bodmer and Bodmer 1974, Colombani and Degas 1973, Dausset and Colombani 1973).

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populations than any other populations yet studied. HL-A9, W28, and W31 are the other common genes. W10, W16, and one of the antigens of the cross-reacting set of HL-A5, W5, and W15, account for the majority of the second series variability.

The HL-A antigens vary geographically among the New World populations. W31 of the first series and W15 of the second series are found at higher frequencies among South American populations, whereas W28, HL-A9, and W5 are found at highest frequencies among North and Central American Indian populations. There appears to be a north-south cline for HL-A9; the highest frequency for any population has been observed in Eskimos. Conversely, HL-A2 is observed at high frequencies in central and southern populations tested but is found at much lower frequencies among Eskimos.


The data available on HL-A antigens in Mongoloid populations other than American Indians indicate that the alleles HL-A2, -9, -10, -11, and W31 of the

Table 5.3. Population Distribution of HL-A Haplotypes

<table>
<thead>
<tr>
<th>Population</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>9</th>
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<th>11</th>
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</tbody>
</table>

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At the International Workshop and Conference on Histocompatibility Testing held at Evian, France in 1972, papers were presented in which a large number of populations had been tested using the same or appropriately compared antisera. Frequencies of haplotypes for each of these populations were estimated and the most common haplotypes shown in tabular form (Bodmer and Bodmer 1974, Dausset and Colombani 1973). We have rearranged these data in Table 5.3 to show how the native American populations tested could be readily distinguished from most other populations, including the closely related Mongoloid and Oceanic groups. In this table high (>0.1) and intermediate (0.05-0.01) frequencies are indicated. From this, it can be seen that there are about 10 haplotypes that occur at these levels only in native Americans or at most in one other population. Furthermore, there are many haplotypes that occur commonly in one or more other population(s) but not at all (or very rarely) in native Americans. From this, the discriminating power of the HL-A haplotypes is apparent; it would be a very useful tool for comparing eastern Asian populations with native Americans.

The Gm System

The Gm system resembles HL-A in genetic complexity and immunologic importance. Gm factors contribute to the inherited variations of the heavy chains of IgG. The genetics of the Gm system is complex and similar to that of the Rh red blood cell antigens. The frequencies of some of the Gm factors vary strikingly among populations, and some of the Gm haplotypes (also called allotypes) are distinct to specific populations and characteristically either present or absent in certain populations. This makes the Gm system a most useful population marker. There is presently, however, a number of problems that make it difficult to use these factors to their full potential. As more antisera were developed, more factors were determined. In earlier studies only one or two factors may have been identified, and in subsequent studies four or five factors may have been tested. This makes interpopulation comparisons difficult. Furthermore, Szathmary et al. (1974) have pointed out the difficulties of comparing data because of the use of different varieties of antisera (“Ragg” and “Snag”) which may have caused differences of classifications.

Early in the investigations the population associations of single factors were described (Grubb 1970). Gm(1) is found at nearly 100 percent frequency in non-European populations: Gm(6) is rare except in Negroes and Gm(16) is rare except in Japanese (Van Loghem 1970).

Haplotype combinations have also been used to identify populations. Mongoloid markers reported in the literature are Gm(1,17,21), Gm(1,2,17,21), Gm(1,13,17), Gm(1,11,16), Gm(1,3,5,13,14), Gm(1,3,5,11), Gm(1,11,13,15,16,17,1,5),
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Gm(1,13), Gm(1,13,15,16,17), Gm(1,10,11,17,23), Gm(1,4,5,10,11,14,22,25), and Gm(1,4,22). Caucasoid markers include Gm(1,17,21), Gm(1,2,21), Gm(5,13,14), Gm(4,5,13,14), and Gm(4,5,8,10,11) (Daveau et al. 1975, Giblett 1975, Grubb 1970, Steinberg 1969, Steinberg et al. 1974, Szathmary et al. 1974).

There are certain Gm factors thought to be characteristic of American Indian populations. Factors 1; 1,2; 1,2,21; and 1,21 are the most frequently and ubiquitously found. The Mongoloid marker 1,11,16, however, has been identified among populations of Ojibwa, and Szathmary et al. (1974) reports that Schanfield identified Gm(1,11,16) in an Indian group in the Valley of Mexico and in the Papago of the American Southwest.

The Gmi system has promising potential for distinguishing native American populations from others, if comparison and “control” populations are studied with the same reagents in the same laboratory.

Red Blood Cell Antigens

The red blood cell antigens are the most extensively and intensively tested of the human polymorphisms. Specific gene frequency distributions for American Indians have been known since the earliest investigations of the red blood cell antigens. These have been thoroughly reviewed recently by Mourant et al. (1976) and will be presented only briefly here. In the ABO system, O occurs in extremely high frequencies. In fact, it is assumed that a great majority of prehistoric Indians were of the O blood type and that the presence of A and B usually indicates genetic admixture with nonnative Americans. Native American populations are characterized by a high frequency of M of the MNSs system and, in particular, by the alleles MS and Ms. The frequency of the R2 gene of the Rh system is higher than anywhere else in the world and the frequency of R0r is extremely low or absent. The Kell antigen was probably absent in precontact Indians and occurs in extremely low frequency or is absent in most contemporary American Indian populations. There is an extremely high frequency of Fy* of the Duffy system and the Diego gene (Di*) occurs only in native American, Asian, and some Oceanic populations and, in general, is absent from other populations. There is in general a cline of increasing frequency of the Di* gene from north to south in the Americas. The Lewis A and Lutheran A are thought to occur in American Indian populations only as results of genetic admixture.

Serum Protein Polymorphisms

A large number of serum protein polymorphisms is now known and in some cases alleles found only (or rarely), in Mongoloid populations and in native Americans have been identified (Mourant et al. 1976). The transferrin variant “D Chinese” is found in people of east Asia and also in many American Indian populations but rarely in Europeans or other populations. Transferrin B0-1 has
been reported only in people of Asiatic origin (including Finns, Lapps, and Hungarians) and in native Americans.

The rare Gc variant, Gc Chippewa, has been found so far only in Chippewa and Ojibwa Indians (Szathmary et al. 1974).

The serum lipoprotein systems (Ag, Lp) have been used only occasionally in native American population studies, and no generalization can be made about their distribution at present. However, the Ag system is determined by alleles segregating at four or more closely linked loci and complex combinations of "haplotypes" are known. If populations can be tested with the same antisera using the same techniques, then it is likely that interesting and useful distinctions between populations can be made.

Conclusions

On the basis of the currently available data, there are several polymorphic systems and/or alleles that appear to be unique to native American populations. These include the polymorphisms at the albumin locus, several of the haplotypes of the HL-A system, probably some of the haplotypes of the Gm system, and certain fairly rare alleles (such as Gc Chippewa) of other systems. There is also a large number of new polymorphic traits the distributions of which are not known but which may in due course be shown to have characteristics that make them useful for distinguishing native Americans from other populations.

Using these traits, and others with distinctive frequencies in native Americans, a study could be designed to identify contemporary Asian populations that might have ancient affinities with native Americans.

Acknowledgments

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References

Blood Polymorphisms


Localization of the amino acid substitution site in a new variant of human serum albumin, albumin Mexico-2

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Contributed by Baruch S. Blumberg, February 4, 1980

ABSTRACT Using an electrophoretic screening procedure, we have discovered that two species of human serum albumin occur that are indistinguishable by conventional electrophoretic methods. We suggest that these species be referred to as albumins Mexico-1 and Mexico-2. Isolation and determination of the partial sequence of the cyanogen bromide fragment of albumin Mexico-2 that differs from the corresponding fragment of the common albumin revealed this variant to arise from at least a glycine/aspartic acid substitution at position 550. This region of the albumin molecule is involved in the binding of the fatty acid, palmitate.

At least two dozen electrophoretic variants of human serum albumin have been reported (1, 2), but only two of them have been characterized with respect to their primary structures: albumin A (the common albumin) and albumin B, a rare variant found mainly in people of European origin (2, 3).

We have previously reported the existence of an albumin variant (albumin Mexico) that possesses a lower electrophoretic mobility than albumin A at pH 8.6 (4). This variant is relatively common in Indians of Mexico and the southwest United States and reaches polymorphic frequencies in the Pima Indians of Arizona and other Indians (5, 6).

The standard methods for analyzing albumin variants are limited to the detection of surface charges. We have developed a more sensitive screening procedure that involves cleavage of the albumin with cyanogen bromide and analysis of the fragments by polycarylamide gel electrophoresis in the presence of nonionic detergent (7). Using this method, we have found that two species of albumin Mexico exist that are indistinguishable by conventional techniques. We suggest that these species be termed albumins Mexico-1 and Mexico-2. We have isolated a cyanogen bromide fragment of albumin Mexico-2 that differs from the corresponding albumin A fragment and identified the molecular difference by peptide mapping and sequence determination.

MATERIALS AND METHODS

Variant Screening. Albumin was cleaved at the methionine residues by treating 25 μl of serum with 50 mg of cyanogen bromide in nitrogen-saturated 70% formic acid overnight followed by lyophilization (repeated once from water). The fragments were reduced in 300 μl of 0.2 M triethanolamine-acetate buffer (pH 8.6), containing 8 M urea, 25 mM methyl amine (cyanate trap), 60 mM dihydrothreitol, and 10 mM EDTA for 4 hr at 37°C. The peptides were then alkylated to prevent mixed disulfide formation by adding iodoacetamide to a concentration of 130 mM and reacting for 30 min in the dark at room temperature. The reaction was stopped by addition of excess dithiothreitol. The samples were diluted 1:5 and precipitated by addition of trichloroacetic acid to 20%. The fragments were collected by centrifugation, washed with acetone/0.2% HCl and acetone, and dried under reduced pressure. All solutions contained 1% thiodiglycol during precipitation. Each sample was then dissolved in 300 μl of loading solution (8 M urea/1% thiodiglycol/20 mM dithiothreitol) and reduced for about 90 min at 37°C before the gels were loaded.

The fragments (load equivalent to about 2 μl of serum) were resolved on 12% polyacrylamide gels containing 5 M urea, 6 mM Trition X-100, and 5% acetic acid (7). The same gel conditions were also used for isolation of the variant cyanogen bromide fragments in a preparative gel electrophoresis apparatus.

For comparison, conventional 8% polyacrylamide gels were run with native, uncleaved sera and the Tris/EDTA/borate buffer of Peacock et al. (8) or 50 mM Tris-HCl (pH 8.6).

Peptide Analysis. The protocol has been described in detail (9). Briefly, purified cyanogen bromide fragments were digested with trypsin in 0.1 M ammonium bicarbonate (pH 8.1) at a 1:25 enzyme-to-substrate ratio. The incubation was made 0.2 mM in tosylphenylalanylchloromethyl ketone (TPCK) to inhibit residual chymotryptic activity. The insoluble trypsin cores were recovered and digested with thermolysin.

Peptides were resolved by microfingerprinting on Avicell coated plates as described (9) except that electrophoresis was for 90 min. Peptides were visualized with fluorescamine, eluted with and hydrolyzed in 6 M HCl, and subjected to amino acid analysis on a Durrum (Palo Alto, CA) D-500 amino acid analyzer.

Edman Degradation. The manual procedure was performed essentially as described by Edman (10) except that the coupling and cyclization times were doubled and 30% pyridine was used as the aqueous phase (in place of 0.1 M HCl) due to the insolubility of the peptide in the latter. The resulting amino acid thiazolinones were converted to free amino acids by hydrolysis in 6 M HCl containing 0.1% SnCl₂ for 8 hr at 150°C (11).

Materials. Sera. Only samples previously classified by cellulose acetate electrophoresis (Tris citrate buffer, pH 5.4) as being heterozygous or homozygous for the albumin Mexico allele were subjected to the new screening procedure. Eight samples were from the San Carlos Apaches of southeastern Arizona; seven of these were heterozygotes for albumin Mexico-1, as were two samples of unspecified origin. One of the Apache samples was an albumin Mexico-2 heterozygote. Seven samples were from the Pima Indians of the Gila River Community in Arizona; five were found to be heterozygotes for albumin Mexico-2 and two were homozygotes. Two of the heterozygotes were the parents of one of the homozygotes.
Reagents. Electrophoresis-grade acrylamide was from Bio-Rad. Ultrapure urea was obtained from Bethesda Research (Rockville, MD). Cyanogen bromide, fluorescamine, thioglycol, trifluoroacetic acid, and iodoacetamide were purchased from Pierce. TPCK-treated trypsin was from Worthington and thermolysin was obtained from Sigma. Dithiothreitol was from Calbiochem. Phenylisothiocyanate was from Beckman. Pyridine and ethyl acetate used for sequence determination were from Burdick and Jackson (Muskegon, MI).

RESULTS

Fig. 1 compares the electrophoretic mobilities in an 8% polyacrylamide gel of intact, undenatured sera containing albumin phenotypes A/Mexico-1, A/Mexico-2, Mexico-2/Mexico-2, A/B, and A/A. The heterozygotic albumins A/Mexico-1 and A/Mexico-2 possess two bands of equal intensity, one migrating identically to the common albumin (A/A) and one migrating more slowly. The albumin Mexico-2 homozygote has a single band that comigrates with the slow component of both types of albumin Mexico heterozygote. Albumin A/B, shown for comparison, again has one band comigrating with albumin A/A and another band migrating even more slowly than the albumins Mexico.

In order to differentiate between variants that migrate similarly in conventional electrophoretic systems but that may arise from amino acid substitutions in different regions of the albumin molecule, we devised a screening strategy. A small quantity of whole serum was treated with cyanogen bromide to cleave at each of the six methionine residues of albumin. The fragments were then reduced, alkylated, and resolved on polyacrylamide gels containing urea, acetic acid, and Triton X-100. In control experiments (not shown) we found that other serum proteins do not interfere with these analyses. Purified, defatted albumins (3, 12) produce the same pattern. Fig. 2 shows the results obtained by treating the same samples shown in Fig. 1 in this manner. The albumin Mexico-2 heterozygote shows two bands of about equal intensity in the vicinity of fragment CNBr VII (residues 549–585), one comigrating with the normal fragment and one possessing a greater mobility (fragment CNBr VII M2). The homozygote of albumin Mexico-2 exhibits only the faster migrating variant fragment. These results are indicative of a substitution site in fragment CNBr VII of the variant molecule. Heterozygotic albumin B, shown for comparison, possesses a band in the normal position of fragment CNBr VII and a very fast migrating band, CNBr VII B, consistent with its postulated lysine/glutamic acid substitution at position 570 (3). The cyanogen bromide fragments of albumin Mexico-1 migrate identically to those of albumin A under these conditions. Thus, the molecular difference between albumins Mexico-1 and A, as indicated by the differences in electrophoretic mobilities of the native proteins (Fig. 1), could not be identified by this system.

In order to localize the amino acid substitution site within fragment CNBr VII of albumin Mexico-2, it was necessary to isolate this fragment in a homogeneous state. This was accomplished by preparative polyacrylamide gel electrophoresis in the presence of urea, acetic acid, and Triton X-100. Fig. 3A shows the elution profile of a preparative gel run obtained from 400 µl of cleaved and alkylated whole serum from an individual homozygous for albumin Mexico-2. Fragment CNBr VII M2 eluted first, followed by fragments CNBr VI, V, I, and III and uncleaved CNBr II and III. Fig. 3B shows an analytical gel run of the fragments purified by preparative electrophoresis.

Fig. 1. Electrophoretic mobilities of native variants. Track 1, A/Mexico-1; track 2, Mexico-2/Mexico-2; tracks 3 and 4, A/Mexico-2 (parents of individual in track 2); track 5, A/B; track 6, A/A. Gel was 8% polyacrylamide containing 50 mM Tris-Cl (pH 8.6). Stain was Coomassie brilliant blue R-250.

Fig. 2. Resolution of alkylated cyanogen bromide fragments of albumin variants. Gel 1, A/A; gel 2, A/Mexico-1; gel 3, Mexico-2/Mexico-2; gel 4, A/Mexico-2 (parent of individual in gel 2); gel 5, A/B. Gels were 12% polyacrylamide containing 5 M urea, 6 mM Triton X-100, and 5% acetic acid. Stain was Amido black 10 B. Cyanogen bromide fragments were originally identified on the basis of molecular weight and amino acid composition and are numbered by Roman numerals in accordance with their order in the known sequence of albumin A. M2 and M refer to variant peptides found only in albumins Mexico-2 and B, respectively; m refers to modifications probably arising from oxidation of unreacted cysteine or lysine alkylation by iodoacetamide. Fragments CNBr II and IV have not been identified.
Fragment CNBr VII and VIIM2 were digested with trypsin. Peptides that precipitated out during tryptic digestion were recovered and digested with thermolysin. Resolution of the peptides was then accomplished by microfingerprinting. In Fig. 4 the tryptic peptide maps of fragments CNBr VII and VIIM2 are compared. The major difference is the absence of peptide T1 in albumin Mexico-2. Upon clution and amino acid analysis of the peptides, it was found that peptide T1 from CNBr VII was homologous in composition with residues 549–557 in albumin A, which has the sequence Asp-Asp-Phe-Ala-Ala-Phe-Val-Glu-Lys (13). Peptide T1a from CNBr VIIM2 was also homologous in composition with residues 549–557, but lacked one aspartic acid residue and contained one glycine residue (Table 1). The remainder of the tryptic peptides of albumin Mexico-2 were homologous in composition to that expected from the known sequence of albumin A (13). This was in some cases also confirmed by peptide mapping and analysis of the thermolysin peptides (data not shown). The spot migrating just below peptide T5 in CNBr VII appears to be a mixture of derivatives of peptides T1 and T5. Thus, albumin Mexico-2 appeared to arise from the substitution of a glycine for an aspartic acid residue at position 549 or 550.

Due to the presence of two aspartic acid residues in tandem at the NH2 terminus of fragment CNBr VII, it was necessary to perform two cycles of the Edman degradation on this fragment to determine which aspartic acid residue was substituted. The first cycle released residues in the ratios: aspartic acid, 1.00; glycine, 0.16; and leucine, 0.14. Cycle two produced glycine.
is possible that in some cases this is because the “variant” arises from some of the artifacts mentioned above. In other cases, this could be due to neutral/acidic residue substitutions which would not be detected at pH 3.0 unless the Triton-binding properties were altered. (For albumin Mexico-1, the latter is more likely.) It is conceivable that albumin Mexico-1 also possesses the glycine/aspartic acid substitution found in Mexico-2 but that it is obscured by a second substitution.

Incomplete cleavage by cyanogen bromide is responsible for the appearance of a major band migrating above fragment CNBr III (Fig. 2). This band has a molecular weight of about 23,000 on sodium dodecyl sulfate gels and probably arises from the known partial cleavage of the methionine-cysteine bond at position 123 [fragments CNBr II + III (18)].

Screening for variants of albumin (or other macromolecules) by the method described here could be of great aid to future anthropological studies. The origins and affinities of certain groups of American Indians have been studied through the distributions of albumins Naskapi and Mexico (2). These distributions are consistent with linguistic and archæological evidence of population movements and contacts (2). However, albumin studies can be misleading in cases where two variants comigrate in conventional electrophoresis systems (such as cellulose acetate or starch gel) but are not molecularly identical. The two Mexico variants illustrate this point. Albumin Mexico was first reported in 1967 (4) and since that time has been found in American Indian groups from the southwestern United States of Guatemala (2). Because all of these surveys have used conventional electrophoresis techniques, it is impossible to determine what proportion of samples classified as Mexico is Mexico-1 or Mexico-2. Triton electrophoresis of cyanogen bromide fragments of eight serum samples from the San Carlos Apaches and two samples of unspecified origin that had been classified as heterozygous for albumin Mexico showed only one of them to have the fast migrating CNBr VII fragment characteristic of albumin Mexico-2. Thus far, the variant has been observed in seven Pima Indians—the homozygous female and her heterozygous parents reported here and in an unrelated male homozygote and three unrelated heterozygotes. Additional surveys will be necessary to establish the distributions and gene frequencies of both albumins Mexico-1 and Mexico-2. Such studies may shed additional light on the population relationships of Indian groups in the Southwest and Mexico.

The literature on human serum albumin reports over 80 “named variants,” but only about two dozen distinct electrophoretic mobilities are detectable at three different pH values on starch gel electrophoresis (1). The use of Triton electrophoresis of cyanogen bromide fragments may alleviate this confusion in two ways. First, it may distinguish between variants that comigrate on conventional gels but have different primary structures by localizing substitution sites to particular regions of the molecule. Second, the acidic, denaturing, and reducing conditions under which the gels are run eliminate many artifacts expected in serum samples suffering from harsh treatment (aggregation, deamidation, oxidation, etc.). Artifacts of this nature may account for some of the published reports of albumin variants.

A further advantage of the Triton method is its ability to resolve neutral substitutions as well as charged ones by virtue of differential Triton binding to regions of proteins possessing high helical probability (15). We have resolved several histone variants differing only in the substitution of one or two neutral amino acids by this method (16, 17). This allows the possible detection of a new class of albumin variants that could not be recognized on conventional systems.

Some named variants (including albumin Mexico-1) exhibit the same pattern as the common albumin A in our system. It

**DISCUSSION**

The results reported here indicate that albumin Mexico-2 is a new variant, differing from albumin A by the substitution of glycine for aspartic acid at position 550. This loss of one negative charge is consistent with the lower anodic electrophoretic mobility of the native protein at pH 8.6. It is of interest that fragment CNBr VII is also the site of the only other known substitution, the glutamic acid → lysine change occurring at position 570 in albumins Oliphant and Ann Arbor [which are electrophoretically indistinguishable from albumin B (3)]. Our finding of a more positively charged fragment CNBr VII in albumin B supports the notion that these three variants are the same protein (3, 14). We have found electrophoretic differences in cyanogen bromide fragments I, V, and VI in other albumin variants (not shown).

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Some named variants (including albumin Mexico-1) exhibit the same pattern as the common albumin A in our system. It

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues per mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>2.0</td>
</tr>
<tr>
<td>Thr</td>
<td>0.0</td>
</tr>
<tr>
<td>Ser</td>
<td>0.0</td>
</tr>
<tr>
<td>Glu</td>
<td>1.0</td>
</tr>
<tr>
<td>Pro</td>
<td>0.0</td>
</tr>
<tr>
<td>Gly</td>
<td>0.0</td>
</tr>
<tr>
<td>Ala</td>
<td>2.0</td>
</tr>
<tr>
<td>Val</td>
<td>1.0</td>
</tr>
<tr>
<td>Met</td>
<td>0.0</td>
</tr>
<tr>
<td>Ile</td>
<td>0.0</td>
</tr>
<tr>
<td>Leu</td>
<td>0.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.0</td>
</tr>
<tr>
<td>Phe</td>
<td>2.0</td>
</tr>
<tr>
<td>His</td>
<td>0.0</td>
</tr>
<tr>
<td>Lys</td>
<td>1.0</td>
</tr>
<tr>
<td>Arg</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* From J. R. Brown (13).
† Uncorrected for hydrolysis loss.

1.0, aspartic acid, 0.22; and alanine, 0.18. It is thus aspartic acid 550 that has been replaced by glycine in albumin Mexico-2.

**Table 1. Amino acid composition of peptides T1 and T1a from fragments CNBr VII and VIIM2, respectively**

**Amino acid** | **Residues per mole** | **Peptide T1** | **Peptide T1a**
--- | --- | --- | ---
Asp | 2.0 | 1.9 | 1.0
Thr | 0.0 | 0.0 | 0.1
Ser | 0.0 | 0.0 | 0.2
Glu | 1.0 | 1.1 | 1.3
Pro | 0.0 | 0.0 | 0.0
Gly | 0.0 | 0.0 | 1.1
Ala | 2.0 | 2.0 | 2.2
Val | 1.0 | 1.0 | 1.0
Met | 0.0 | 0.0 | 0.0
Ile | 0.0 | 0.0 | 0.0
Leu | 0.0 | 0.0 | 0.2
Tyr | 0.0 | 0.0 | 0.0
Phe | 2.0 | 2.0 | 1.8
His | 0.0 | 0.0 | 0.0
Lys | 1.0 | 1.0 | 1.0
Arg | 0.0 | 0.0 | 0.1

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currence of albumin variants and diabetes in individual persons may provide interesting results.

We thank Drs. Peter Bennett, David Pettite, and Lowell Weitkamp for providing some of the serum samples used in this study and Ms. Judy Mamas for technical assistance. This work was supported in part by Grant RR-05539 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, by Grants CA-06551, CA-06927, and CA-15155 from the National Institutes of Health, and by an appropriation from the Commonwealth of Pennsylvania.

Alloalbuminemia in North India

HARSURINDER KAUR,1,2 SAMUEL G. FRANKLIN,1 PRABODH K. SHRIVASTAVA,2 AND BARUCH S. BLUMBERG1

SUMMARY

Electrophoretic screening of sera from 550 individuals from Punjab, North India, revealed four cases of alloalbuminemia. Two albumin variants migrated slower and two migrated faster than the common albumin A. These variants were further analyzed by electrophoresis of their cyanogen bromide fragments to localize their molecular differences. One of the slow variants appears similar to, if not identical with, albumin B, with an altered cyanogen bromide fragment CNBr VII. The other slow variant appears to be a new variant (proposed name albumin Punjab) differing from albumin A in an altered fragment CNBr VI (which also occurs in albumins Kashmir and Adana) and in an altered fragment CNBr I. Among the fast variants, one has the same altered fragment CNBr V as albumin Naskapi, while the other appears to be a new variant (proposed name albumin Patiala) having an altered fragment CNBr V1. The presence of albumin Naskapi in Punjabis, North American Indians, and Eti Turks (previously reported) is consistent with the existence of a common ancestral population in which the mutation to Naskapi occurred before the migrations eastward and westward.

INTRODUCTION

Atal et al. [1] reported the first case of alloalbuminemia in India in a patient from Agra in Uttar Pradesh, North India. On cellulose acetate electrophoresis, one...
band migrated in the position of the common albumin A and the other migrated more slowly. McDermid [2] described slow alloalbuminemia in two individuals from Delhi. This variant was provisionally identified as albumin Gainsville by McDermid but was later found to be indistinguishable by electrophoretic mobility from albumin Afghanistan [3]. Albumin Kashmir, a slow heterozygote, was found in London in a Muslim family originally from Kashmir by Tarnoky and Dowding [4]. It was found to be identical in terms of mobility with albumin Afghanistan described by Weitkamp and Buck [5]. Three more slow variants were found in India in the recent past [6-8].

The first case of homozygous alloalbuminemia of the fast type in Indian populations was reported by Sehajpal et al. [9] from Punjab. We previously described a variant in a Tharu tribal from Uttar Pradesh where the albumin resolved into two fractions, one migrating with albumin A and the other faster than it. This was apparently a case of transient alloalbuminemia [10] because when examined a few months later the albumin in a fresh sample from this individual appeared normal. We detected yet another atypical albumin in an individual from Leh [11]. In this case, the albumin separated into two fractions located within the confines of the albumin A zone. Rao et al. [12] reported two more cases of alloalbuminemia from Hyderabad, one of which was found to be identical with albumin Kashmir, and another migrating faster than it was designated albumin Koya Dora after the name of the tribe in which it was discovered. Figure 1 shows the areas where albumin variants were found in different population groups.

We report here on four albumin variants found among 550 individuals from the state of Punjab. By using a technique we developed to more clearly discriminate between variants with similar electrophoretic mobilities [13], we find that two of the variants are similar to or identical with albumins B and Naskapi and that the other two appear to be new variants.

**MATERIALS AND METHODS**

**Population**

The material for the present study includes 100 Jat Sikhs and a heterogeneous group of 450 other Punjabis (table 1). Punjab is a flat alluvial plain with an altitude less than 300 m. The state is thickly populated with a density of about 269 persons per sq km. The Punjabis are physically well built, of medium to fair complexion, and in many respects resemble Europeans. They are considered to be distinct from other Indian population groups. Ethnically they appear to be a mixture of diverse populations, of which Nordic, Mediterranean, and Scythian are thought to be the major ones [14]. The Jat Sikhs are a dominant agricultural community of Punjab and are thought to be of Indo-Scythian origin.

**Methods**

About 5 ml of venous blood was drawn from each individual under sterile conditions. The samples were examined by the techniques of Poulik [15], Fine [16], and Jeppsson et al. [17], and by use of 8% polyacrylamide gels using 0.05 M Tris-HCl buffer, pH 8.6.

Sera with variant albumins were further analyzed by first cleaving the samples with cyanogen bromide followed by alkylation of the resulting polypeptides with iodoacetamide and electrophoresis in 12% polyacrylamide gels containing 5% acetic acid, 5 M urea, and 6 mM Triton X-100, pH 3.0 [13]. In some cases, samples were also run in 15% polyacrylamide sodium dodecyl sulphate gels using a method modified from that of Laemmli [18].
RESULTS

Four different types of alloalbuminemia were found in this study. Two migrate slower than albumin A and were tentatively designated albumins Sirhind and Punjab; the other two migrated more rapidly and were designated albumins Khan- na and Patiala. Albumin Patiala appeared to be a homozygote by electrophoretic criteria. However, in the absence of family data, the possibility that the Patiala allele is heterozygous with an allele producing little or no albumin product cannot be ruled out. For the purposes of discussion and the calculation of gene frequency, we consider it to be homozygotic. All the other variants in this study were heterozygous with albumin A. These variants were compared with standard variants of which B, Kashmir, Adana, Makiritare, Mexico, and Santa Ana were slow and Naskapi, Mersin, Paris, and Gent were fast.

Figure 2 shows that albumin Sirhind migrates like albumin B in 8% polyacrylamide gels. The other slow variant, designated albumin Punjab, has essentially
ALLOALBUMINEMIA

TABLE 1

DISTRIBUTION OF ALBUMIN VARIANTS IN PUNJAB, NORTH INDIA

<table>
<thead>
<tr>
<th>Tribe</th>
<th>No. tested</th>
<th>Variant type</th>
<th>No. variants</th>
<th>Gene frequency</th>
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<tbody>
<tr>
<td>Jat Sikhs</td>
<td>100</td>
<td>Punjab</td>
<td>1</td>
<td>.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>2</td>
<td>.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Patiala*</td>
<td>1*</td>
<td>.010</td>
</tr>
<tr>
<td>Khatris</td>
<td>76</td>
<td>B</td>
<td>1</td>
<td>.007</td>
</tr>
<tr>
<td>Balmikis</td>
<td>109</td>
<td>B</td>
<td>1</td>
<td>.005</td>
</tr>
<tr>
<td>Banias, blacksmiths, carpenters, etc.</td>
<td>265</td>
<td>Naskapi</td>
<td>1</td>
<td>.002</td>
</tr>
</tbody>
</table>

* Homozygote (by electrophoresis).

the same electrophoretic mobility as albumins Kashmir and Adana in this gel system. Albumin A/Khanna migrates like A/Naskapi, whereas albumin Patiala/Patiala migrates faster than Naskapi/Naskapi.

Variants that have the same mobilities in conventional gels may actually arise from substitutions in different regions of the molecule. By localizing the substitution to a particular cyanogen bromide fragment, a higher level of discrimination between albumin variants can be achieved. This technique also minimizes the effects of deamidation, oxidation, and aggregation.

Figure 3 compares the CNBr fragments of the variants from figure 2. Albumin Sirhind, which comigrates with albumin B in 8% gels, shows the same variant CNBr fragment VI B as does albumin B. Albumin Kashmir appears to have a similar CNBr fragment VI as albumin Adana (residues 447-548). Albumin Punjab, which has the same electrophoretic mobility as albumins Kashmir and Adana in conventional polyacrylamide gels, also has a similar fast-moving fragment VI (fragment VIAd) and, in addition, has an altered CNBr fragment IP (residues 1-87).

Albumin A/Khanna shows the same altered fragment CNBr V as A/Naskapi (VN, residues 330-446). Albumin Patiala shows an apparent deletion of fragment VI on Triton gels. However, when this sample was subjected to SDS polyacrylamide gel electrophoresis (both cleaved and uncleaved), it behaved as did albumin A. This indicates that fragment VI is not deleted and that a substitution in fragment VI has probably shifted the mobility to that of another fragment.

DISCUSSION

Two new variants have been found in this study, which we have designated albumins Punjab and Patiala. Albumins Kashmir and Adana (which we described in a population from Turkey: the Eti [19]) appear to be identical with each other within the limits of resolution of the methods used in this study. The name albumin Kashmir is formally precedent. Albumin Sirhind appears to be the same as albumin B and albumin Khanna the same as Naskapi. This is the first report of what appears to be albumin B in India. Previously, this variant has been reported only in Europeans [20]. Albumin Khanna appears identical with albumin Naskapi,
Fig. 2.—Electrophoretic mobilities of native albumin variants. Tracks 1 and 11, A/A; track 2, A/Naskapi; track 3, A/Khanna; track 4, A/B; track 5, A/Sirhind; track 6, A/Punjab; track 7, A/Adana; track 8, A/Kashmir; track 9, Naskapi/Naskapi; track 10, Patiala/Patiala. Na, Naskapi. Pa, Patiala. Electrophoresis was in 8% polyacrylamide gels containing 50 mM Tris-HCl, pH 8.6. Stain was Coomassie Brilliant Blue R-250.

which, heretofore, has been found only in North American Indians and the Eti Turks [19, 20].

Albumin Punjab, apparently a new slow variant that comigrated with albumins Kashmir and Adana in 8% polyacrylamide gels, showed altered CNBr fragments I P (residues 1-87), which include the binding sites of Cu**, Ni**, and Ca**, and VI Ad (residues 447-548), which includes the binding sites of tryptophan and palmitate. We earlier observed a similar shift in fragment CNBr I (data not shown) in a slow Pima variant and found that it had arisen because of deletion of the N-terminal aspartic acid.

The slow variant, albumin Kashmir, has been reported to occur in people originating from three different areas (Kashmir, New Delhi, and Hyderabad) of India [2, 4, 12]. Albumin Adana was identified in 1980 [19] in the Eti Turks of southeastern Turkey. In our present study, both albumins Adana and Kashmir show a similarly altered fragment CNBr VI (residues 447-548) when analyzed by the new screening procedure. The possible presence of this rare gene among certain Indians and Turks is consistent with a common origin for these populations and with the occurrence of the mutation to albumin Kashmir prior to their separation.

Albumin Patiala appears to be a new variant that differs in fragment CNBr VI, the principal binding site of palmitate [21]. This is the same fragment in which the variations for albumins Kashmir and Punjab occurs, but the amino acid substitution appears to be different from any other population. This is the fastest migrating variant reported so far.

The apparent presence of albumin B in North India indicates that this gene is not restricted to European populations as it previously appeared to be. According
to historical and other information, the Punjabis descended from early Aryan settlers who migrated from Europe, entered India on the northwestern border, and established themselves in Punjab [14]. They avoided union with aboriginal populations. In contemporary Punjab, there is a subpopulation that has certain physical characteristics similar to those of Europeans. The albumin B variant may have been introduced by this group. Alternatively, it may have been introduced by the British who first entered India in 1599. India, after 1857, was under British rule until 1947. Population admixture is known to have occurred during this period.

Since most of the variants have been reported from North India, which was invaded by Afghans, Moghuls, Turks, and Arabs in early historic times, it appears reasonable to postulate that some of the albumin variant genes entered India from central Asia. This may explain the presence of what appears to be albumin Naskapi in this population. This hypothesis is further supported by the finding that albumins Kashmir and Adana appear to be identical, that is, that the populations share a variant not found elsewhere. The Naskapi variant was detected first in an American Indian tribe, the Naskapi Indians of Quebec, in high frequency [22]. It is

Fig. 3.—Resolution of alkylated cyanogen bromide fragments of albumin variants. Gel 1, A/A; gel 2, A/Naskapi; gel 3, Naskapi/Naskapi; gel 4, A/Khanna; gel 5, A/B; gel 6, A/Sirhind; gel 7, A/Punjab; gel 8, A/Adana; gel 9, A/Kashmir; gel 10, Patiala/Patiala. Electrophoresis was on 30-cm-long, 12% polyacrylamide gels containing 5 M urea, 6 mM Triton X-100, and 5% acetic acid. Stain was Amido Black 10 B. Fragments are numbered in accordance with their order in the known sequence of albumin A, P, N, Ad, and B refer to variant peptides found only in albumins Punjab, Naskapi (Khanna), Adana (Kashmir, Punjab), and B (Sirhind), respectively. m refers to modifications probably arising from oxidation of unreacted cysteine or lysine alkylation by iodoacetamide. Fragment IV has not been identified. Unlabeled minor bands near the bottom of gels 5 and 6 arise during storage and may be due to proteolysis.
also found in lower frequency in other North American Indians, including the
Athabascan-speaking people of western North America [20] who probably mi-
grated to the New World many centuries ago from locations in Asia. In 1980 we
reported the presence of albumin Naskapi in Eti Turks [19]. The contemporary
distribution of albumin Naskapi in Asia and in North America could be explained
by its origin in central Asian people who later migrated into India, Turkey, and
the Americas.

ACKNOWLEDGMENT

We are grateful to Dr. Lowell R. Weitkamp for the albumin Kashmir sample.

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MISCELLANEOUS
The Selective Solvation of the Hyaluronic Acid Complex of Ox Synovial Fluid

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(Received 6 February 1956)

This work was undertaken to test the suggestion made by Johnston (1955) that a large part of the volume occupied in solution by the coiled-chain particles of the hyaluronic acid complex (Ogston & Stanier, 1951, 1952) might be 'impenetrable' by serum albumin. Such an impenetrability could equally be regarded as a positive selective solvation of the hyaluronic acid complex by components of the solution (water and buffer salts) other than albumin (Ogston, 1954). This hypothesis was tested by studying the effect of concentration of albumin on the sedimentation of the hyaluronic acid complex in the ultracentrifuge; this is the method used by Lauffer and his colleagues (see Lauffer, Price & Petre, 1949) to estimate the 'solvation' of virus particles.

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The rate of sedimentation of the hyaluronic acid complex becomes zero when the reciprocal of its effective specific volume (i.e. its effective density in solution) is equal to the density of the solution. Its effective density can be regarded as being determined by the relative proportions in the sedimenting particles of (i) unsolvated hyaluronic acid complex, (ii) solvent free of albumin, (iii) solvent containing albumin, of the same composition as that of the bulk solution.

EXPERIMENTAL

Hyaluronic acid complex. This was prepared by the method of Ogston & Stanier (1950). The complex was washed 16 times with buffer on a sintered-glass filter of average pore diameter 1 μ. The resulting solution showed only a single, very sharp boundary in the ultracentrifuge, and no sign of any free protein.
Serum albumin. Ox serum (50 ml.) was diluted with 50 ml. of water and mixed with 100 ml. of a saturated solution of ammonium sulphate. The precipitated globulins were removed by centrifuging. The supernatant solution was mixed with 150 ml. of saturated solution of ammonium sulphate/100 ml. of supernatant. The precipitated albumin was filtered off, dialysed thoroughly against water and freeze-dried.

Separate stock solutions of hyaluronic acid complex and of albumin were made and dialysed against buffer [0-1 m-NaCl, 0-01 m-NaH2PO4, 0-01 m-KH2PO4 (pH 7-3)]. These two solutions and buffer were mixed to give a final concentration of hyaluronic acid complex of 2-8 mg/ml. and varying final concentrations of albumin. A solution containing sucrose was made by dissolving a weighed amount of sucrose in a measured volume of the dialysed solution of hyaluronic acid complex.

Sedimentation. Sedimentations were observed in a Svedberg oil-turbine ultracentrifuge by the method of Cecil & Ogston (1948) with the modified bar-schlieren optical system of Baldwin (1953). The temperature was about 27° and the speed of rotation 900 rev./sec.

RESULTS

Sedimentation of hyaluronic acid complex in albumin solutions. Except at very low concentration the rate of downward sedimentation of the hyaluronic acid complex is less than that of albumin (Johnston, 1955). It is therefore not possible to observe the downward sedimentation of the complex in the region of constant concentration of albumin. However, an upward-moving boundary of the complex, originating at the bottom of the cell, should be observed if the concentration of albumin is high enough.

With the lower concentrations of albumin (6 and 9 %, w/v) an ascending boundary of hyaluronic acid complex located itself within the region of raised concentration of albumin at the bottom of the cell, at a level at which the density of the solution is equal to the reciprocal of the effective partial specific volume of the complex (Fig. 1A). This boundary moved up the cell only as albumin accumulated at the bottom of the cell (Fig. 2).

When the concentration of albumin was raised to 12 %, (w/v) the peak moved upwards more rapidly, through the uniform concentration of albumin (Fig. 1C, Fig. 2). The step in the albumin base-line seen in Fig. 1C at the position of the ascending boundary of complex is due to the effect of the hyaluronic acid on the rate of sedimentation of the albumin (Johnston, 1955), which causes the concentration of albumin to be greater below the boundary of the complex than above it (Johnston & Ogston, 1946).

Sedimentation of hyaluronic acid complex in sucrose solution. Fig. 1D shows that no ascending boundary of the complex was observed in 15 % sucrose (w/v). A descending boundary occurred, within the descending gradient due to the sucrose; integration of the gradient of the refractive index due to the sucrose, between the base-line (15 %, w/v) and the position of the descending boundary of the complex, by using the specific refractive increment of sucrose, showed that the concentration of sucrose at this point was 14 % (w/v).

Fig. 1. Schlieren diagrams showing the sedimentation of hyaluronic acid complex in the presence of serum albumin. Sedimentation occurs from right to left. (A) Complex (2-8 mg/ml.) in albumin (6 g/100 ml.); 40 min. after reading full speed; schlieren angle 10°. A boundary of the complex (c), in the accumulation of albumin at the bottom of the cell, appears at the extreme left; at the right (from left to right) are the descending boundaries of albumin (a) and of the complex (c'), and the meniscus (m). (B) Control, albumin (6 g/100 ml.) with no complex; 95 min. after reading full speed; schlieren angle 10°. No boundary is seen at the extreme left in the accumulation of albumin at the bottom of the cell, appears at the extreme left; at the right (from left to right) are the descending boundaries of albumin (a) and of the complex (c'), and the meniscus (m). (C) Complex (2-8 mg/ml.) in albumin (12 g/100 ml.); 110 min. after reading full speed; schlieren angle 10°. A boundary of the complex (c) is seen at the extreme left ascending through uniform concentration of albumin (compare A, and see text). (D) Complex (2-8 mg/ml.) in sucrose (15 g/100 ml.); 30 min. after reading full speed; schlieren angle 12°. No ascending boundary of complex is visible; at the right the descending boundary of complex (c) is seen in the gradient due to sedimentation of the sucrose (see text).
DISCUSSION

The results show that the hyaluronic acid complex sediments downwards in concentrations of albumin less than 9%, but upwards in 12% albumin, and indicate that it would sediment at zero rate in about 10% albumin. Extrapolation of Johnston’s (1955) indirect observations on the sedimentation of the complex in lower concentrations of albumin supports this conclusion. The density of the solution containing 10% albumin is 1.033; that of buffer solution without albumin is 1.008; the partial specific volume of the complex is 0.69 (Ogston & Stanier, 1951). The resultant value for the positive selective solvation by solvent free of albumin, or a volume impenetrable by albumin, is 12 ml/g. of hyaluronic acid complex.

This quantity is equivalent to the ‘impenetrable volume’ defined by Johnston (1955), but its value is very much less than the 1000 ml/g. which Johnston’s hypothesis demands. This must be taken as disproving Johnston’s hypothesis, and some other explanation is needed of the effect of hyaluronic acid complex on the sedimentation of albumin, on which the hypothesis was based.

Even so, the value of 12 ml/g. may appear large at first sight. It is, however, of the order to be expected from simple steric considerations (Schachman & Lauffer, 1949) based on the coiled-chain nature of hyaluronic acid (Ogston & Stanier, 1951, 1952; Blumberg & Oster, 1954; Laurent & Gergely, 1955). Since the complex sediments downwards in a solution of sucrose of concentration 14% (w/v), whose density is 1.052, its selective solvation in this solution must be less than 6.5 ml/g. Schachman & Lauffer (1949) found a similar difference with tobacco mosaic virus in solutions of albumin and of sucrose.

The physiological consequence of this finding is that molecules of the size of serum albumin should be able readily to penetrate solutions or gels containing hyaluronic acid, such as are found in intercellular spaces.

SUMMARY

1. The sedimentation of hyaluronic acid complex (of ox synovial fluid) has been observed in solutions containing various concentrations of serum albumin. The hyaluronic acid sediments downwards in concentrations of albumin of 9 g/100 ml and below, but upwards in a concentration of 12 g/100 ml.

2. Hyaluronic acid complex will therefore sediment at zero rate in a uniform concentration of albumin of about 10 g/100 ml. This indicates a selective solvation of the complex by solvent free of albumin, or a volume impenetrable by albumin, of about 12 ml/g. of hyaluronic acid.

3. This quantity is far lower than that suggested by Johnston (1955).

4. The selective solvation is considerably less in sucrose.

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Addendum

A NOTE ON THE VEGETATION
OF THE NORTHERN ISLETS OF RONGELAP ATOLL, MARSHALL ISLANDS, MARCH 1959
B.S. BLUMBERG* AND R.A. CONARD

Fosberg\textsuperscript{1,2} reported changes in the vegetation of the northern islets of Rongelap Atoll (observed in 1956) which he inferred might have been associated with the radioactive fallout that occurred on this atoll in 1954. During the medical survey of the Rongelap people\textsuperscript{3} carried out in March 1959, an opportunity arose to visit some of these islets and to re-examine the vegetation. A helicopter was available for transportation, which permitted general and detailed air examination as well as two short ground surveys. The northern islets were estimated to have received a radiation dose of \( \approx 3000 \) r. The islets of Naen and Gegen were examined in greatest detail. The most striking feature observed from the air was the generally gray color of much of the vegetation, in contrast to its

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Figure A-1. Map of Rongelap Atoll showing position of major islets.
normal green color. Ground surveys revealed that *Scaevola sericea* was common and normal in appearance. Many of the *Guettarda speciosa* appeared to be in poor condition (Figure A-2). In some, all or nearly all the leaves were gone from the terminal 1 to 12 in. of the branches, and other leaves were yellowed and shriveled. In other *Guettarda*, nearly all the leaves were gone, and the bushes appeared completely dead. More than 50% of the *Guettarda* were affected in whole or part. In one area of Naen several hundred yards inland from the ocean beach, there was a field of ≈30 *Guettarda*, all of which were dead. Some young *Pisonia grandis* were seen which appeared to be in good condition. Mature *Pisonia* were seen which were partially defoliated, but these did not appear to be greatly different from those seen on Rongelap Islet on the southeast corner of Rongelap Atoll. None of the mistletoe-like clumps described by Fosberg were observed. Several *Ochrosia oppositifolia* were seen with nearly complete defoliation, which appeared dead. A small grove of coconut trees near the center of Naen Islet contained 4 to 5 dead trees within a radius of ≈300 yards, which were decapitated at heights 5 to 12 ft above the ground with no evidence of axe or machete marks. Two 2-headed coconut trees were seen, one with fronds that were mostly brown and appeared dead growing from the trunk ≈2 ft below the true crown of the tree. Several trees had dry and shriveled fronds, and ≈6 had deformed bulges 4 to 8 ft below the crown with apparently normal growth above the bulges.

Photographs of the affected vegetation were examined by Dr. Fosberg, and he stated that the changes were similar to those he had previously reported.

It is not possible to evaluate the cause of the changes from the present observations. More extensive and detailed botanical and ecological surveys will be necessary, both on the islands that received radiation and on those that did not, to determine whether the changes seen bear any relation to fallout. In particular, it should be noted that these observations were made during the dry season.

We are indebted to Professor Frank Richardson of the University of Washington for identifying the plants, and to Commander W. Lyons, USN, for his assistance in taking the photographs.

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Coalescence of Caudal Vertebrae in the Giant Dinosaur Diplodocus

By Baruch S. Blumberg and Leon Sokoloff

The coalescence, seen frequently in the caudal vertebrae of the giant dinosaur Diplodocus longus, has been re-examined. A comparison with vertebral fusion in contemporary species suggests that the changes in the dinosaur were of traumatic rather than rheumatoid type.

Degenerative joint disease occurs widely in macrosomic species of mammals regardless of their position in the taxonomic scale. Its recognition in paleontologic specimens dates back to Virchow's observations of spinal osteophytosis in the cave bears of Bonn in 1895. The skeleton, unlike the soft tissue of these ancient animals, fossilized and the pathologic changes were preserved. An instance of coalescence of caudal vertebrae in the dinosaur Diplodocus was described by Osborn and interpreted by Moodie as "spondylitis deformans". This observation has found its way into authoritative textbooks on rheumatic diseases as evidence that atrophic (rheumatoid) arthritis occurred in fossil reptiles. The present paper describes the occurrence of vertebral coalescence in six specimens of Diplodocus. These findings suggest that such union of vertebral bodies may have been more frequent than is popularly supposed. The observations are of interest in the comparative pathology of degenerative spinal disease because of the magnitude of the weight-bearing problem in these giant species.

Material

Although several dozen specimens of Diplodocus have been excavated, they have for the most part been incomplete or fragmented. This was due either to natural causes during the long period of their earthly interment or damage at the time of excavation. In only ten instances were sufficient caudal vertebrae available for analysis. Seven of these 10 specimens have been inspected by us (USNM 10865, CM 84, CM 94, AMNH 223, AMNH 493, AMNH 608, AMNH 655, table 1). Information concerning the others has been obtained from published accounts or by personal correspondence with curators of museums.

Description of normal caudal vertebrae: Diplodocus longus had at least 73 caudal vertebrae (fig. 1). It was distinguished from the other giant sauropods by its long whip-like tail. Only the proximal and middle portions (up to approximately caudal body 20) were of considerable size. The distal vertebrae were small. The cervical vertebrae of Diplodocus were opisthocoelous (i.e., had a well developed ball and socket configuration, the ball facing cephalad), unlike modern reptiles that generally have a procoelous spine (the ball facing caudad). In contemporary reptiles, the joints between the vertebral bodies (centra)
are of diarthrodial type and have no intervertebral disc. Nevertheless, in view of the poor
development of the ball and socket configuration in the Diplodocus, we may speculate that
an intervertebral, notochordal disc may have existed. The reconstruction of Holland7
and Gilmore8 suggests that the vertebrae rested on the ground somewhere between Nos.
20 and 27, and beyond.

Description of lesions: In eight of the specimens (all but AMNH 493 and CM 84), a
major portion of the middle segments of the caudal vertebrae was preserved. Three of these
had coalescence of vertebral centra in the region of vertebrae 15 to 21. The coalescence
was characterized by total annular ankylosis. The lesion was not smooth and symmetrical,
but irregular and knobby. In specimen AMNH 655, a post-fossilization fracture of the
site of ankylosis permitted the insertion of a probe into the intervertebral space revealing
that the bony union occurred only in the region of the “annulus fibrosus”; i.e., the union
was peripheral only. Incorporated into the ankylosic intervertebral mass were the chevron
bones (ventral hemal arches) of the respective intercentra. In two additional specimens
(AMNH 223, AMNH 516), fusion of the chevron bone occurred in the absence of the
coalescence of the vertebral centra. The zygapophyseal joints were ankylosed in two of
the specimens in the areas of vertebral coalescence. In these two, the dorsal interspinous
ligaments also were involved (fig. 2).

The most common vertebra affected was caudal No. 20. In one animal (CM 94), only
one interspace was fused; in a second (AMNH 655), three were affected; and in the
third (USNM 10865), four segments were involved. In the latter two, the involvement was
not continuous—an intervertebral space was skipped.

In CM 84, in which only the 12 proximal caudal segments were present, coalescence
occurred in segments 2-3, but this appeared to be somewhat different from the other speci¬
mens in that the ankylosis affected only the centra. However, only the proximal 12 verte¬
brae were preserved, and observations were not possible in the region of vertebra 20. The
musculotendinous ossification seen in Tyrannosaurus and duck-billed dinosaurs (Trachodon)
and other species9,10 was not seen in Diplodocus, with the exception of the dorsal inter¬
spinous ligaments already referred to. Degenerative arthritis of the peripheral joints was
not seen in the specimens where these joints were preserved (AMNH 223, USNM 10865,
CM 84, CM 94*).

Discussion

Vertebral Coalescence in Contemporary Species

The gross character of the coalescence in Diplodocus differs somewhat from
the principal patterns observed in contemporary species. For purposes of com¬
parison with the fossil lesion, the contemporary sorts are described and
grouped into four types.

1. Normal structural coalescence: In the distal thoracic vertebrae of many
species of birds, there occurs a fusion of the margins of the centra (vertebral
bodies) and the zygapophyseal joints, associated with ossification of inter¬
spinous ligaments (fig. 3). The resulting rigidity is regarded as of functional
advantage in flying.11 Ossification of tendons, resembling that of the spinal
ligaments, also occurs in some avian species: e.g., turkeys and cranes.

2. Congenital anomalies: A variety of sporadic and hereditary fixed deformi¬
ties of the tail is encountered in some strains of mice.19 In some instances, the
principal defect lies in a disproportion between the length of the tendons and the
caudal vertebrae; ankylosis and deformity of the latter result from the
abnormal positioning of the bodies with respect to each other. In other in-
Table 1.—A List of Excavated Specimens of Diplodocus longus in Which a Reasonable Number of Caudal Vertebrae Are Available

<table>
<thead>
<tr>
<th>Specimen no.*</th>
<th>Vertebral coalescence</th>
<th>Inter-central joints</th>
<th>Zygapophyseal joints</th>
<th>Chevrons</th>
<th>Interspinous ligaments</th>
<th>Osteophyte formation</th>
<th>Vertebrae present</th>
<th>Location discovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMNH 223 (6)</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>First six caudals; reference (6) shows other bones now missing</td>
<td>Como Bluffs, Aurora, Wyoming</td>
</tr>
<tr>
<td>AMNH 655 (3)</td>
<td>17–18</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Middle caudals</td>
<td>Bone Cabin Quarry, Wyoming</td>
</tr>
<tr>
<td>AMNH 516 (3)</td>
<td>16–18</td>
<td>0</td>
<td>0</td>
<td>Coalescence to centra</td>
<td>0</td>
<td>0</td>
<td>Anterior caudals</td>
<td>Bone Cabin Quarry, Wyoming</td>
</tr>
<tr>
<td>USNM 10865 (8)</td>
<td>17–20</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Complete vertebral column</td>
<td>Dinosaur National Monument, Utah</td>
</tr>
<tr>
<td>C.M. 84 (7,16)</td>
<td>2–3</td>
<td>Yes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12 proximal only</td>
<td>Sheep Creek, Albany Co., Wyoming</td>
</tr>
<tr>
<td>C.M. 94 (7)</td>
<td>20–21</td>
<td>Yes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>“Between 20–30 caudals”</td>
<td>Sheep Creek, Albany Co., Wyoming</td>
</tr>
<tr>
<td>C.M. 307 (7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>38 posterior caudal. Includes from just before middle to top of tail</td>
<td>Red Fork of Powder River, Johnson Co., Wyoming</td>
</tr>
<tr>
<td>C.M. 662 (7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>33 caudals from middle</td>
<td>“Wyoming”</td>
</tr>
<tr>
<td>AMNH 493</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25 distal</td>
<td>Bone Cabin Quarry, Wyoming</td>
</tr>
<tr>
<td>AMNH 608</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>26 caudals: middle, distal, and proximal</td>
<td>Bone Cabin Quarry, Wyoming</td>
</tr>
</tbody>
</table>

†At present in the Museum of the Senckenbergische naturforschende Gesellschaft, Frankfurt a. M., W. Germany. (Personal communication: Dr. W. Struve, 1960; and Mrs. B. Nicholls, 1960.)
‡At present in Cleveland Museum, Cleveland, Ohio. (Personal communication: Black C., and Roberts, D. C., 1960.)
Fig. 1.—Reconstruction of a skeleton of Diplodocus longus. (Photograph courtesy of the U. S. National Museum, Washington, D. C.)

stances, the bony changes represent incomplete segmentation rather than acquired ankylosis. Hereditary coalescence of cervical vertebrae can also occur in mice. Sporadic incomplete segmentation has been reported in the dorsal vertebrae of salamanders and other amphibians. In man, with the exception of rare anomalies such as Klippel-Feil syndrome, congenital variations in vertebral coalescence are generally confined to the lumbo-sacral region (congenital "block" vertebrae).

3. Inflammatory spondylitis: Vertebral ankylosis may result from infectious processes and, in man, from Marie-Strümpell disease as well. In the latter, vertebral fusion occurs both by ankyletic repair of the apophyseal joints and ossification of the annulus fibrosus of the intervertebral discs. Characteristically, continuous segments of the vertebral columns and the sacroiliac joints are affected. Involvement of the spinal ligaments is seen occasionally.

4. Degenerative osteophytosis of the spine: With the exception of "kissing osteophytes" and the senile kyphotic spine, ankylosis is not a feature of degenerative spinal disease in man. However, it occurs with some frequency in several quadruped mammalian species. The condition known as ankylosing spondylitis in the dog probably falls into this classification, and similar changes are seen in horses and lower primates. Little is known of the state of the apophyseal joints in these conditions. The published illustrations indicate that it differs from Marie-Strümpell disease in the absence of predominant diarthrodial inflammation, in the localized rather than extensive involvement of the vertebral column, and sparing of the sacroiliac joints. The ankylosis in
Fig. 2.—Coalescence of caudal intervertebral spaces Nos. 17–19 in Diplodocus, U. S. National Museum, No. 10865. The spaces between the vertebral bodies (centra) are occupied by bone. The bifid structures on the ventral aspect of the intervertebral spaces are the chevron bones. The latter also are ankylosed to the intervertebral bony mass. The zygapophyseal joints are fused in these vertebrae. The interspinous ligaments attached to the dorsal process of vertebrae Nos. 18 and 19, are partially ossified. (Photograph courtesy of U. S. National Museum.)

the quadrupeds apparently results from blending of osteophytes with ossification of paraspinal ligaments at the margins of the vertebral bodies.

Vertebral Coalescence in Diplodocus

Any consideration of the nature of the vertebral union in fossil material is necessarily speculative.

1. Normal structure: The possibility that the coalescence was a natural structural feature in the Diplodoci should be considered. Since the fusion was found in only 6 of the 10 specimens, it was not a universal and therefore characteristic feature of this species. If the animals with the coalescence represented a subspecies, one might expect that they would be localized to a limited region of their range. That this is not so can be seen from table 1. Despite these observations, it is conceivable that this variation represented a genetic polymorphism; that is, the occurrence of two or more inherited forms of a species in a geographic area. Presumably there might have been a difference in the survival values of each of the forms. The distribution of the several vertebral types in this species population might thus have resulted from the ecologic balance of these values. Another possibility is that one form was of value in
one environmental circumstance, but a change in the environment resulted in selection in favor of the second type. *Diplodocus*, for example, is thought to have been semi-aquatic. With the drying-up of swimming places, more time would have been spent on land. The additional strength imparted to the tail by the coalescence might conceivably have been of value to the animal in resting a portion of its huge bulk on the ground. The latter view was proposed originally by Hatcher. Subsequently this thesis was regarded as untenable by Gilmore because the revised reconstruction of the animal placed the resting place of the tail in a more distal position.

It should also be noted that spotty ossific transformation of cartilaginous structures could have occurred in different individuals of this species as it does in contemporary animals. Thus, ossification of epiphyses takes place in rodents to varying degrees at different rates throughout life, subject both to genetic and sex influences. The sex of dinosaurs has not been ascertained from the skeletal remains with perhaps isolated exceptions cited in the French
Fig. 4.—Coalesced cervical vertebrae from *Mus musculus* (DBA/2J X STR/IN)F2. There is a partial bony union of the vertebral bodies without other deformity. The apophyseal joints are normal.

...as that of birds, has been found regularly in certain bulky dinosaurs, notably *Trachodon*. Nevertheless, coalescence of vertebral bodies in *Diplodocus* was not associated with this finding, and the analogy to the avian vertebral fusion falls down here. Against the possibility that this is a natural rather than a pathologic process are the patchy distribution of the changes, with skipping of isolated segments, the exophytic appearance, and the fact that occasional ankylosis of ribs to vertebrae was present in at least one specimen (USNM 10865).

Coalescence of cervical and caudal vertebrae has also been recorded in other sauropods.

2. *Congenital anomalies*: Most of the congenital, anomalous coalescence of vertebrae in other species occurs in the lumbo-sacral region. In only one of the specimens (CM 84) was the coalescence near the sacral vertebrae. Because of the distance from the sacrum and the other reasons listed above, it appears unlikely that the changes observed in the other animals could be classed as congenital anomalies. In passing, it is interesting to note that the illustration of the sacrum of the *Diplodocus* AMNH 516 shows only partial coalescence of primary sacral segments 2 and 3, suggesting that the sacral...
region was subjected to individual variation in this species, as it is in mice and men.

3. Inflammatory spondylitis: The lesion differs from that of Marie-Strümpell disease in that the distribution is patchy, the sacroiliac joints are spared, and in its exophytic character. Moodie classified these dinosaur lesions as "spondylitis deformans," a term sometimes employed for Marie-Strümpell disease, although others have used it for degenerative spinal disease. Pemberton, however, classified them as atrophic (rheumatoid) spondylitis. Although some of the chevron bones were deformed, the characteristic destructive lesions of infectious arthritis were not seen in these specimens.

4. Osteoarthritis: The most likely possibility is that the coalescence in Diplodocus is a traumatic osteoarthritis. It is sharply localized and an exophytic rather than destructive lesion. It is not unlikely that the mechanical trauma of dragging the tail across the ground, or predation by other animals could have caused the changes. Although ankylosis is not characteristic of degenerative spinal changes in man, it is seen with some frequency in quadrupeds.

Although degenerative arthritis was seen in early Eocene mammals, it is remarkable that degenerative disease of the peripheral joints was not seen in these huge reptiles, despite the obvious problem of weight bearing. This is of interest in relation to the role of weight bearing in osteoarthritis in general. Since dinosaur bones had no epiphyses, it in conceivable that the joints were
covered by a specially modified, thick cartilage. Peripheral osteoarthritic erosion may have occurred in the cartilage but did not extend into the bone and therefore were not preserved. In contemporary reptiles, no or minimal epiphyseal development is seen. It also is conceivable that reptiles generally are not susceptible to osteoarthritis. Degenerative joint disease has not been studied systematically in contemporary reptiles. We have, however, observed osteoarthritis affecting many diarthrodial joints in a two year old male Cuban iguana (*Cyclura m. macleayi*, fig. 5).

**SUMMARY AND CONCLUSIONS**

The coalescence, seen frequently in the caudal vertebrae of the giant dinosaur *Diplodocus longus*, has been re-examined. A comparison with vertebral fusion in contemporary species suggests that the changes in the dinosaur were of traumatic rather than rheumatoid type.

**ACKNOWLEDGMENTS**

Our studies were facilitated by many individuals and agencies to whom we are indebted. The following were of particular help: Mrs. Rachel H. Nichols, American Museum of Natural History, New York, N. Y.; Dr. Doris M. Cochran, U. S. National Museum, Washington, D. C.; Dr. Wolfgang Struve, Senckenbergische naturforschende Gesellschaft, Frankfurt a. M., Germany; Dr. David C. Roberts, Cleveland Museum of Natural History, Cleveland, Ohio; Dr. Craig Black, Carnegie Museum, Pittsburgh, Pa.; Dr. Rainer Zangerl, Chicago Natural History Museum, Chicago, Ill.

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CAUDAL VERTEBRAE COALESCEIN IN DIPLODOCUS


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Bioethical Questions Related to Hepatitis B Antigen

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ABSTRACT

Blumberg, Baruch S.: Bioethical questions related to hepatitis B antigen. Am J Clin Pathol 65:848-853, 1976. The bioethical problems that are raised from the studies of hepatitis carriers can be viewed as a conflict between public health interests and individual liberty. When the risk to the public is clear, and the restrictions on personal activities are small, there is little problem in arriving at appropriate regulations. For example, the transfusion of blood containing hepatitis B antigen is a disadvantage to the patient recipient and it has been stopped. The denial of the right to donate blood is not a great infringement of personal activity and the individuals concerned and society have agreed to accept this moderate restriction. The problems raised by person-to-person transmission are more difficult. The extent of the hazard to the public is not clear, since it is not (now) possible to distinguish carriers who transmit disease from those who do not. On the other hand, if all carriers are treated as infectious, the hazards imposed on the carrier may be enormous, i.e., loss of job and ability to continue in the same profession, restriction of social and family contacts, etc. What is clear is that for a very large number of carriers, the risk of transmitting hepatitis by person-to-person contact must be very small. Until we know more about this problem and have developed methods for distinguishing carriers who are likely to transmit hepatitis and those who are not, we should not cast all carriers into the same stigmatized “class.” (Key words: Bioethics; Hepatitis B carriers.)
The test for Australia antigen (that is, hepatitis B surface antigen, HBsAg) was described in 1964, and after we showed its association with hepatitis in 1967, it was rapidly applied to the screening of blood donors. Okochi and Murakami in Japan, and Gocke and his colleagues, soon found that the "carriers" identified by this test could transmit hepatitis by blood transfusion more readily than individuals who did not have detectable antigen in their blood. This led to the nearly universal practice of testing donor blood prior to its transfusion, and this has resulted in a decreased frequency of posttransfusion hepatitis due to hepatitis B.

It has always been clear that hepatitis B may be transmitted by means other than transfusion. Hence, consideration had to be given to the transmission of hepatitis from the carriers to others with whom they came in contact, i.e., family, co-workers, friends, etc. This has resulted in what may be an unprecedented situation in medicine and in society. The "carrier" is not apparently ill (although there may be detrimental, chronic effects on the liver) and is not aware that he is different from others until this occult character is determined by a blood screening test. He is thought to be able to contaminate others with whom he comes in contact, particularly those with whom he is most intimate. Patients with leprosy, active tuberculosis and other infectious diseases are ill and require treatment. They usually are aware of their disease. The hepatitis carrier is not obviously ill and learns of his state only after screening. People can be occult carriers of typhoid organisms, staphylococcus, and other agents, but a very small proportion of the population is tested to identify carriers of these infectious agents, whereas, because of blood donor screening, millions of people are tested each year to see whether they are hepatitis carriers. Asymptomatic carriers of syphilis, gonorrhea, and other venereally transmitted organisms are in a somewhat similar situation, but they transmit the disease primarily through sexual contact, and they can be treated, whereas hepatitis may be transmitted by a variety of other mechanisms, and the carrier, once identified, cannot at present be treated.

At the outset it should be said that we do not have detailed or quantitative information on which carriers can transmit hepatitis, or the extent of the hazard they present. Carriers probably differ in infectiousness, but the characteristics that determine this are not well known. For example, Heathcote and colleagues have suggested that "carriers" who also have evidence of active hepatitis are more likely to transmit hepatitis than are carriers who have no evidence of disease. Theoretically, it would be expected that carriers with a relatively high concentration of the large (Dane) particles (which are thought to be the infectious virus particles of hepatitis B) would be more likely to transmit hepatitis than those without detectable Dane particles, but, to my knowledge, this has not been demonstrated consistently. It has also been stated that carriers who also have the "e" antigen are more likely to be infectious, and this hypothesis is now being tested. It is clear that the appropriate epidemiologic studies have not been done, and much of the evidence for transmission of disease from asymptomatic carriers rests on anecdotal information. There have been several published reports and some unpublished stories of dentists, physicians, nurses and other health personnel who have transmitted hepatitis to patients with whom they came in contact. In at least one study of health personnel carriers it was concluded that transmission of hepatitis B did not occur.

There have been several studies from which it can be concluded that clinical hepatitis, antibody to HBsAg, and the carrier state cluster in families. However, the information on transmission gained
from the study of families cannot be
directly extrapolated to other social groups
since the spread is controlled not only by
simple transmission but probably by ge-
netic factors as well. (The ability to become
a carrier, develop the disease, or develop
antibodies may be affected by genetic
control.)

Hence, we can tentatively conclude that
under appropriate circumstances, some
carriers can transmit disease. However,
the risk is not enormous and is conceivably
amenable to control measures. Independ-
ent of the actual data that are available,
or in spite of lack of substantial evidence,
carriers are in many cases regarded as
hazardous sources of disease. The myth
is at present stronger than the facts and
will probably remain so until more knowl-
dge is available. There are no docu-
dmented studies of the social and personal
consequences of being identified as a car-
errier. My colleague, Dr. Jana Hesser, is con-
ducting an intensive study to obtain infor-
mation on the unusual and sometimes
unfortunate consequences of identification
as a carrier.

Some carriers have been forced to leave
their work as nurses, physicians, hospital
and laboratory workers when they were
identified as carriers. I was once asked in
a country with a high frequency of carriers
whether regulations should be passed to
prevent the admission of carriers to medici-
val school and to officer training school.
(I advised against this.) It has been recom-
mended that nurses be screened for HBs-
Ag at the time of their entry into the
profession and that those who are found to
be carriers should be advised to “discon-
tinue nursing as a career.”11 This would
be a difficult decision for an eager entrant
into a profession for which he or she has
worked hard for many years. It has been
suggested that health personnel should be
routinely screened. Should this be comp-
ulsory or voluntary? Should a person be
forced to take a test that might cause him
to lose not only his immediate job but also
the chances for other jobs in his profession?

On the other hand, what are the risks that
a health worker frequently exposed to pa-
tients with hepatitis or HBsAg will become
ill or become a carrier, and how are these
risks communicated and prepared for? We
have studied the patients and staff of a
renal dialysis unit, located in Philadelphia,
which has a high rate of hepatitis infection
among the patients. Over the course of
about four years there were 184 staff
employees of this unit. Of these, seven
developed hepatitis, but only one became a
carrier. Hence, less than 1% of those ever
exposed became a carrier. Additional data
regarding this unit are given below.

Collateral studies, published some years
ago, give an indication of the maximum
carrier rate to be expected in individuals
who have had extensive exposure to hepa-
titis B infection.13 These include drug
addicts, prisoners (many of whom were also
drug addicts), and patients who had re-
ceived blood transfusions with donor blood
from commercial sources which had not
been screened for HBsAg. In each case
about 4% of the people exposed became
carriers. There is some evidence that the
propensity to become a carrier after being
exposed to infection may be under genetic
control,2 and if this is the case, the
gene frequency in the population would
determine the upper frequency of carriers
to be expected. In our society the devel-
opment of the carrier state, even among
people with extensive exposure, is unlikely
to exceed about 4%, and usually would
be much less. (This estimate does not hold
for special groups of patients with defective
or impaired immune systems.)

Some additional problems that do not
have obvious solutions at present have
arisen. The frequency of hepatitis B is ex-
tremely high in Vietnam, Thailand and
elsewhere in Southeast Asia, where large
d NUMBERS OF SERVICEMEN FROM THE UNITED
States were stationed. Hepatitis is one of
the most common causes of morbidity in these populations, and there are data to show that there is an increased number of carriers in the United States as a result of the experience in Vietnam. If it is a detriment to be classified as a carrier, is the military responsible for compensation of the carrier even though he is not obviously sick? The psychological impact of being classed as a carrier, possibly associated with loss of occupation, ostracism by fellow workers, and change in social habits, may be sufficiently detrimental to constitute a disability, and this will depend on the social attitudes that develop in the community as information about carriers becomes widespread. Carriers have been referred to as "lepers" in the medical press (the author of the article condemns this practice); newspaper articles about carriers appear occasionally in the public press. An attitude towards carriers is developing but, again, little is known about public attitudes towards hepatitis. It is not generally a calamitous disease, such as cancer, nor is it as benign as the common cold. How much do people fear it, if at all? How do they view social, professional or sexual contacts with carriers or people with the disease?

Another potentially serious problem has come up in connection with adoption of children. HBsAg carriers are more common among Vietnamese (about 6%) than among native Americans and north Europeans (about 0.1–0.5%). In a European country Vietnamese orphans who were being considered for adoption were, among other things, tested for HBsAg. Some were found to be carriers. Should this be a deterrent to their adoption? Should this single test determine the course of a young life without any adjudication and without any real knowledge of the potential danger the child might impose?

A series of particularly difficult problems in relation to hepatitis has arisen in renal dialysis (artificial kidney) units. Fifty percent or even more of individuals treated in certain units may chronically harbor hepatitis B virus and have HBsAg in their peripheral blood. This is frequently accompanied by a chronic, anicteric hepatitis. Epidemics and sporadic cases of acute hepatitis, usually icteric and sometimes severe, have affected the staffs of these units, and deaths have occurred. There has been considerable effort to keep hepatitis out of dialysis units, and in some places one of the criteria for rejection for treatment has been the presence of HBsAg in the patient's blood. If no other renal dialysis unit is available in the community, this is tantamount to allowing the patient to die. Dr. London and others in our laboratory have recently completed a detailed four-year survey of a renal dialysis unit which admits "positive" cases and have obtained quantitative data on the risk to patients and staff on a unit with a high risk of contamination.

These studies will be reported shortly, but can be broadly summarized to say that staff are at a relatively high risk of developing hepatitis (10% per year of exposure), but a very small number will become carriers (1 of 184). The dialysis patients have a high risk of becoming chronic carriers. Such patients do not develop acute icteric hepatitis, but they do have mild elevations of their liver enzymes. In some cases these abnormalities persist for long periods. It is not clear how deleterious this is to the patients although it does not appear to affect their mortality adversely. Further, it is likely that epidemiologic control, changes in equipment, procedures and environment can probably considerably decrease the incidences of hepatitis in both patients and staff. A hepatitis vaccine that we proposed some years ago is now being tested and, if it proves efficacious, may help to decrease or eliminate the incidence of hepatitis. These factors (i.e., the measurable and limited risk to staff, the relatively mild disease in the patients, the probable effectiveness of control and prevention) should be weighed against
the decision to exclude a person from a required treatment because he carries HBsAg. A similar problem may also arise if patients who are known to be carriers are denied needed surgery.

A characteristic of many large-scale public health control programs is adverse results that could not have been anticipated prior to the institution of the program. For example, the control of malaria has in many areas resulted in a markedly decreased infant mortality with a large increase in population. When this has not been accompanied by a concomitant increase in food production, the nourishment and wellbeing of the population have actually decreased. The construction of the high dam at Aswan on the Nile has led to the widespread dissemination of schistosomiasis, altered the fish life of the eastern Mediterranean, affected the fertility of the land downstream, and undoubtedly had other effects that will be apparent in coming years.

With the availability of the serologic and environmental tests for hepatitis B, it is now possible to begin the design of control measures for this disease. If the vaccine is found to be effective, then it may also be of value in preventing the development of the carrier state. We are now attempting to investigate the eco-logic biology of the hepatitis B agent to learn whether some of the consequences of control can be known before the program begins. An example, which may or may not ultimately prove to be important, has been studied. In Greece and in the Solomon Islands, Hesser and colleagues have found that HBsAg in parents appears to have an effect on the sex ratio of the offspring. In the Greek study if either parent is a carrier, then there is a distribution of the sex ratio in a direction to increase the frequency of males. In the studies in the Solomon Islands, if the mother is a carrier, there is a larger number of female offspring. If these sex ratio disturbances are a general feature of the carrier state, then elimination of the agent from the environment could change the existing male/female ratios and could have an effect on the sociology of populations.

Summary and Conclusions

Several generalizations can be made from these experiences with hepatitis carriers. The problems that are raised are a result of a conflict between public health interests and individual liberty. When the risk to the public is clear, and the restrictions on personal activities are not great, there is little problem in arriving at appropriate regulations. For example, the transfusion of blood containing hepatitis B antigen is a disadvantage to the patient recipient, and it has been stopped. Removal of "positive" units does not greatly decrease the amount of donor blood. On the other hand, the denial of the right to donate blood is not a great infringement of personal activity, and the individuals concerned and society have agreed to accept this moderate restriction. The problems raised by person-to-person transmission are more difficult. The extent of the hazard to the public is not clear, since it is not (now) possible to distinguish carriers who transmit disease from those who do not. On the other hand, if all carriers are treated as infectious, the hazards imposed on the carrier may be enormous, i.e., loss of job and ability to continue in the same profession, restriction of social and family contacts, etc. Hence, there is a very wide discrepancy between the potential but unknown benefits to society of restricting carriers and the real hazards to the individual carrier.

What is clear is that for a very large number of carriers, the risk of transmitting hepatitis by person-to-person contact must be very small. Until we know more about this problem and have developed methods for distinguishing carriers who are likely to transmit hepatitis from those who are not, we should not cast all carriers into
the same stigmatized "class." It is also likely that technics for preventing transmission can be developed. Cases can be considered on an individual basis in deciding on their disposition, and encouragement given to sponsoring research that will solve the problems of detection and prevention of transmission.

On a broader level, the ethical issue of how much biological knowledge about individuals should impinge on daily lives is raised. Is it appropriate to regulate the risks inherent in people living together and interacting with each other? An issue has been raised with respect to hepatitis because the test can be easily done and because millions of people are tested as part of the blood donor program. As a consequence of these identifications, this particular group of carriers has been identified. There are other forms of carriers, some of them potentially more hazardous (i.e., carriers of staphylococcus, typhoid) who are not routinely tested and therefore not placed at a disadvantage.

It is hoped that many of these problems can be resolved by continued research into the nature of the hepatitis carrier state, and that carriers who have already been identified will not be jeopardized during this period when necessary information is not available.

Acknowledgment. Dr. J. E. Hesser offered helpful discussion of her study of hepatitis carriers.

References

IRON-BINDING PROTEINS, HEPATITIS B VIRUS, AND MORTALITY IN THE SOLOMON ISLANDS

RICHARD G. STEVENS,1 SOLO KUVIBIDILA,1 MARCIA KAPPS,2 JONATHAN FRIEDLAENDER2 AND BARUCH S. BLUMBERG1


The hypothesis that serum levels of ferritin and transferrin are associated with subsequent mortality was tested in a population of Solomon Islanders who had been followed over an 8–12-year period beginning in 1966. A case-control analysis of 105 matched pairs showed that 1966–1970 levels of ferritin were higher and levels of transferrin were lower in Solomon Islanders who had died by 1978 than in matched controls who were alive in 1978. These findings support the hypothesis and, in addition, are consistent with the view that increased iron stores are associated with increased mortality. Among females, the association of ferritin with mortality was more pronounced in chronic carriers of hepatitis B virus than in noncarriers.

ferritin; hepatitis B; iron; mortality; transferrin

Severe iron restriction can result in iron deficiency anemia, which in turn is associated with impairment of immune response and increased susceptibility to infection (1). Fortification of certain foods with iron was mandated in the United States after World War II, presumably in the belief that anemia could be reduced or avoided on a community level. In a series of papers and editorials, Crosby (2) warned of the potential dangers of iron overload as a result of this program of fortification. In particular, he pointed out the dangers of hemochromatosis, as well as other forms of toxicity.

Iron excess may also increase susceptibility to infection (3, 4). An important response of mammals to infection is the decrease of iron absorption in the gut and elevation of temperature. Both of these maneuvers can deny the host's iron to an invading microbe and thus reduce the probability of infection. This phenomenon has been termed “nutritional immunity” by Weinberg (3, 4). Excess body iron stores would short-circuit this defense mechanism.

The Institute for Cancer Research has been studying the relationship of serum iron and iron-binding proteins to infection with hepatitis B virus. In 1974, Sutnick et al. (5) found that serum iron levels were higher among patients with Down's syndrome who were chronic carriers of hepatitis B than among those who were not. A similar observation was made in patients with end-stage renal disease who were receiving dialysis (6), and in apparently normal people from a rural com-
munity in Senegal (Blumberg et al., manuscript). Blumberg et al. (6) found that the elevation in serum iron could not be entirely explained by liver damage as reflected by serum glutamic-pyruvic transaminase (SGPT) levels. They postulated that chronic carriers of hepatitis B virus might be at increased risk of infection and death as a result of increased amounts of available iron.

If elevated iron stores are associated with increased susceptibility to bacterial infection, then increased iron stores should correlate with shortened survival. Since serum ferritin levels normally increase with increasing iron stores, and serum transferrin levels normally decrease, it follows that ferritin should have a direct association with death and transferrin should have a negative association.

In this study, we examined the relationship of serum ferritin and transferrin levels to risk of death in a population of Solomon Islanders who had been followed over an 8- to 12-year period. The association was in the predicted direction. From this it can be inferred that increased iron stores shorten survival, and this can be tested in other populations.

MATERIALS AND METHODS

We tested two hypotheses: 1) the 1966–1970 serum ferritin levels of people who died by 1978 (referred to as Group D) were higher than those of a comparable group of people who survived to 1978 (referred to as Group A); and 2) the serum transferrin levels in Group D were lower than those in Group A.

The study population consisted of six subpopulations of Solomon Islanders first examined during the period 1966–1970 as part of the Harvard-Solomon Islands project (7, 8) originally organized and executed by the late Albert Damon. The overall objective of the project was to define a longitudinal sample of the Solomon Islands population and to investigate the effects of Westernization. A wide range of medical and cultural characteristics, in particular the effect of dietary changes on risk of cardiovascular disease, were determined. At the start of the study, a serum sample was drawn, demographic characteristics were recorded, a medical examination was performed, and anthropometric measurements were taken on each of approximately 2500 individuals. The subjects have been followed over the intervening years. Many have died, and determination of death as of 1978 has been made by anthropologists who have returned to the communities. In one of the subpopulations under study (the Baegu), death status is known as of 1980.

The original sera from the Solomon Islands have been stored since the late 1960s in the blood collections of the Institute for Cancer Research, Philadelphia, at −20 C. The sera were not stored in iron-free containers, and so serum iron determinations were not done. However, iron stores in otherwise normal individuals are directly related to serum ferritin (9–12) and inversely related to serum transferrin (3, 13).

For reasons of economy, we did not undertake a prospective study of the Solomon Island population which would have required the testing of all 2500 sera for iron-binding proteins. A retrospective, matched pairs design was deemed more economical, yet it provided sufficient power to test the hypotheses in question.

A total of 156 deaths had occurred by 1978 among the Solomon Islanders covered in the Harvard-Solomon Islands Project. We matched each death (Group D) with a live control (Group A) for age, sex, subpopulation grouping, and hepatitis B serology. Age and sex are correlated with mortality, and with serum ferritin (14, 15). There were six subpopulations (Nasioi, Kwaio, Lau, Baegu, Nagovisi, and Aita) among the total study population. Mortality experience, diet, and disease load were different among them. (These subpopulations are defined pri-
marily on linguistic grounds. All those villages, or hamlets, that share a common language make up one group, e.g., the Nasioi of Bougainville Island. There is a rough geographic clustering of the Nasioi, but they are distinguished primarily by their language (7). Serum iron levels are known to be higher in carriers of hepatitis B virus (5), and mortality is also correlated with hepatitis B status (16). Hence, the subjects were matched on the serum response to infection with hepatitis B. The serum hepatitis B status was classified in one of the following three categories: 1) negative (no evidence of infection), 2) antibody to hepatitis B surface antigen (anti-HBs) positive, and 3) hepatitis B surface antigen (HBsAg) positive.

Sera were available for transferrin testing in both members of 105 pairs, and for ferritin testing in 94 pairs. There was insufficient serum available from one or both members of the remaining pairs. Some of the sera were depleted after transferrin testing and therefore could not be tested for ferritin.

All 105 pairs were successfully matched on sex. There were 84 pairs successfully matched on population group and 21 that were not. There were 101 pairs matched on hepatitis B status and four pairs that were not. In 86 pairs age was matched to within five years, in six pairs to within 10 but more than five years, and in 13 pairs the difference in age was greater than 10 years.

The cause of death in 51 pairs was unknown. In the remaining 54, the cause was not given in sufficient detail or was not considered medically accurate: seven individuals were reported to have died of "old age," 13 of tuberculosis, 11 of malaria, and so on. These diagnoses were obtained from relatives, friends, or local paramedical personnel; none was obtained from hospital records or autopsy. Since the cause of death is not accurate, it was not used in the analysis.

There is a marked daily variation in iron levels (17), and a smaller variation in serum ferritin levels (18). In this study, we assumed that the time of day the blood was drawn was not correlated with serum levels of iron-binding proteins or with probability of death over the next eight to 12 years.

Serum transferrin was determined using M-Partigen Radial Immune Assay (Behring Diagnostics, La Jolla, CA). Diluted sera are applied to wells cut in a gel containing antibody to transferrin. End-point diffusions are reached within 48 hours and the concentration of transferrin is directly proportional to the diameter of the precipitin ring (19, 20).

Serum ferritin was measured in the laboratory of Dr. Hie-Won L. Hann (Institute for Cancer Research) using radioimmunoassay (Rianen assay kit, New England Nuclear, North Billerica, MA).

Serum HBsAg was determined by immunodiffusion (21) and by radioimmunoassay (22). Antibody to HBsAg subtype AD and to subtype AY was determined by hemagglutination (23).

The other measurements examined in this paper were taken by the staff of the Harvard-Solomon Islands study under the direction of Dr. Damon (7).

**Results**

**Single variable analysis**

We tested the hypothesis that the mean difference in transferrin level among 105 matched pairs of Solomon Islanders (Group D minus Group A level) was less than 0 when a paired t test was used. A Wilcoxon test was also performed to determine if transferrin levels in Group D were less than those in Group A. We applied similar tests to the hypothesis that serum ferritin was higher in Group D than in Group A.

For both transferrin and ferritin, the differences between Group D and Group A were in the direction predicted (table 1), and in both instances the results were
**Table 1**

Comparison of 1966–1970 serum ferritin and transferrin levels of Solomon Islanders dead by 1978 (Group D) and alive in 1978 (Group A)

<table>
<thead>
<tr>
<th></th>
<th>Ferritin</th>
<th>Transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pairs</td>
<td>94</td>
<td>105</td>
</tr>
<tr>
<td>Group D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>62.6 ng/ml</td>
<td>242.4 mg/dl</td>
</tr>
<tr>
<td>Range</td>
<td>2.2–290 ng/ml</td>
<td>109–394 mg/dl</td>
</tr>
<tr>
<td>SEM*</td>
<td>5.4</td>
<td>5.5</td>
</tr>
<tr>
<td>Group A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>51.6 ng/ml</td>
<td>269.4 mg/dl</td>
</tr>
<tr>
<td>Range</td>
<td>1.9–408 ng/ml</td>
<td>120–460 mg/dl</td>
</tr>
<tr>
<td>SEM*</td>
<td>5.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Mean difference</td>
<td>11.0</td>
<td>-27.0</td>
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<tr>
<td>t statistic</td>
<td>1.5</td>
<td>3.2</td>
</tr>
<tr>
<td>One-tailed p value</td>
<td>0.07</td>
<td>0.001</td>
</tr>
<tr>
<td>Wilcoxon rank test</td>
<td>1728.5</td>
<td>1563.0</td>
</tr>
<tr>
<td>One-tailed p value</td>
<td>0.03</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* SEM, standard error of the mean.

significant (p < 0.05) by a parametric test, a nonparametric test, or both.

The mean serum levels of both ferritin and transferrin were within normal limits for both Group D and Group A; ferritin within the range 10–200 ng/ml and transferrin within the range 200–400 mg/dl are considered normal (24). In Group A, the mean serum transferrin level was 269 with a range of 120–460 mg/dl. In Group D, the mean was 242 and the range was 109–394. Mean serum ferritin in Group A was 52 ng/ml with a range of 2–408. In Group D, the mean serum ferritin was 63 and the range was 2–290.

The mean transferrin differences and the mean ferritin differences by sex, age group, and hepatitis B status are shown in table 2. The mean differences between Group D and Group A in serum transferrin were −15 for HBsAg carriers and −30 for noncarriers (negative and anti-HBs); mean ferritin differences were 25 for carriers and 5 for noncarriers.

Although the transferrin differences are uniformly negative (table 2) and most ferritin differences are positive, the ferritin differences vary much more widely than the transferrin differences. In particular, the difference in ferritin among males is 19, yet among females it is only 1 ng/ml.

It is possible that the reduced transferrin in Group D resulted from inadequate nutrition. Measures of nutritional status taken at the time of the original examinations (1966–1970) included triceps skinfold thickness, serum albumin, height, weight, and height/weight ratio. In addition, the following measures taken at the time of the initial examination were also considered: 1) current illness, 2) malaria, 3) gamma globulin levels (which might reflect infection), 4) Hackett spleen size (25), 5) hemoglobin levels, and 6) haptoglobin variant.

There was no significant difference between Group D and Group A in triceps skinfold thickness (in either sex) or in height/weight ratio (table 3), or in reported current illness. Hepatitis B carriers were similar to noncarriers in Groups D and A in height, weight, and height/weight ratio (table 4), regardless of age or sex.

Group D had a small but significantly higher mean amount of gamma globulin than Group A.

There were 39 in Group D and 42 in
### Table 2

Means and mean differences in 1966–1970 serum transferrin and ferritin levels among matched pairs of Solomon Islanders dead by 1978 (Group D) and alive in 1978 (Group A) by age group, sex, and hepatitis B status

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Transferrin</th>
<th>Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of pairs</td>
<td>Group D mean</td>
</tr>
<tr>
<td>0–19</td>
<td>14</td>
<td>243</td>
</tr>
<tr>
<td>20–39</td>
<td>20</td>
<td>236</td>
</tr>
<tr>
<td>40–64</td>
<td>44</td>
<td>239</td>
</tr>
<tr>
<td>65+</td>
<td>27</td>
<td>253</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sex</th>
<th>Transferrin</th>
<th>Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of pairs</td>
<td>Group D mean</td>
</tr>
<tr>
<td>Male</td>
<td>56</td>
<td>242</td>
</tr>
<tr>
<td>Female</td>
<td>49</td>
<td>244</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hepatitis B status†</th>
<th>Transferrin</th>
<th>Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of pairs</td>
<td>Group D mean</td>
</tr>
<tr>
<td>Negative</td>
<td>55</td>
<td>239</td>
</tr>
<tr>
<td>Anti-HBs</td>
<td>21</td>
<td>244</td>
</tr>
<tr>
<td>HBsAg</td>
<td>29</td>
<td>249</td>
</tr>
</tbody>
</table>

* SEM, standard error of the mean.
† anti-HBs, antibody to hepatitis B surface antigen; HBsAg, hepatitis B surface antigen.
Table 3
Differences between matched pairs of Solomon Islanders dead by 1978 (Group D) and alive in 1978 (Group A) in selected individual characteristics measured in 1966–1970

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (range)</th>
<th>Mean difference (D – A)</th>
<th>No. of pairs</th>
<th>p value, two-tailed t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triceps skinfold thickness (mm)</td>
<td>6.8 (3–23) 7.1 (3–18)</td>
<td>-0.26</td>
<td>104</td>
<td>0.33</td>
</tr>
<tr>
<td>Males</td>
<td>5.5</td>
<td>5.7</td>
<td>-0.19</td>
<td>56</td>
</tr>
<tr>
<td>Females</td>
<td>8.4</td>
<td>8.8</td>
<td>-0.35</td>
<td>48</td>
</tr>
<tr>
<td>Height/weight ratio (cm/kg)</td>
<td>3.37 (2–8) 3.31 (2–8.5)</td>
<td>0.06</td>
<td>104</td>
<td>0.28</td>
</tr>
<tr>
<td>Albumin (g/liter)</td>
<td>39.5 (28–49) 41.1 (29–54)</td>
<td>-1.59</td>
<td>67</td>
<td>0.02</td>
</tr>
<tr>
<td>Males</td>
<td>39.1</td>
<td>41.5</td>
<td>-2.3</td>
<td>40</td>
</tr>
<tr>
<td>Females</td>
<td>40.1</td>
<td>40.6</td>
<td>-0.5</td>
<td>27</td>
</tr>
<tr>
<td>Gamma globulin (mg/ml)</td>
<td>50.4 (36–78) 47.5 (31–87)</td>
<td>2.9</td>
<td>67</td>
<td>0.03</td>
</tr>
<tr>
<td>Males</td>
<td>49.0</td>
<td>46.8</td>
<td>2.3</td>
<td>40</td>
</tr>
<tr>
<td>Females</td>
<td>52.1</td>
<td>48.4</td>
<td>3.7</td>
<td>27</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>12.4 (7–18) 13.1 (8–19)</td>
<td>-0.64</td>
<td>105</td>
<td>0.015</td>
</tr>
<tr>
<td>Males</td>
<td>12.7</td>
<td>13.6</td>
<td>-0.9</td>
<td>58</td>
</tr>
<tr>
<td>Females</td>
<td>12.1</td>
<td>12.4</td>
<td>-0.3</td>
<td>47</td>
</tr>
</tbody>
</table>

Group A who scored 0 on the Hackett scale (not palpable). Twenty-eight in Group D and 23 in Group A scored 3 or greater. A Wilcoxon test did not reject the hypothesis of no difference.

Hemoglobin binds 60 per cent of the total body iron, and haptoglobin is the major serum hemoglobin binding protein. Hp 0 indicates that there is little or no haptoglobin. We found that Group A had a significantly higher mean hemoglobin level than Group D (table 3). In Group D, 20 per cent (21/105) had hemoglobin below 11 g/dl, while in Group A only 14 per cent (15/105) were this low. The mean ferritin in Group D with low hemoglobin was 78, and the mean transferrin was 239. The mean ferritin and transferrin in Group A with low hemoglobin were 49 and 273, respectively. Thus, low hemoglobin was not reflected in lowered ferritin, and to the extent that ferritin reflects body iron stores, low hemoglobin in these people was not due to lack of available iron. Haptoglobin variants among Groups D and A are shown in table 5. There were 32 in Group D and 23 in Group A with the Hp 0 variant (none detectable), and this difference is not significant.

Finally, in table 6 the study sample is divided into four groups on the basis of reported year of death. There was follow-up on one of the subpopulations (the Baegu) to 1980. For many, year of death was unknown. For the remaining 80, it is only approximate. As seen in table 6, differences between Groups D and A in transferrin and ferritin are similar in each period. Thus, deaths occurring soon after sera were drawn were similar to deaths occurring long after sera were drawn in regard to the association of transferrin and ferritin with mortality.

From this analysis, we conclude that serum iron-binding proteins are associated with mortality, and the association cannot be explained by differences between Groups D and A in nutritional status or in existing illness at the time the sera were drawn. The difference between Groups D and A in ferritin level is greatest in carriers of hepatitis B.

Logistic model fitting
To study the joint effects of ferritin and transferrin on mortality, and to study interactions among these proteins and other variables, the conditional logistic regres-
### Table 4

<table>
<thead>
<tr>
<th>Height, weight, and height/weight ratios for Solomon Islanders dead by 1978 (Group D) and alive in 1978 (Group A) by age, sex, and hepatitis B status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group D</strong></td>
</tr>
<tr>
<td>0-19 years</td>
</tr>
<tr>
<td>Males</td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>17</td>
</tr>
<tr>
<td>18</td>
</tr>
<tr>
<td>22</td>
</tr>
<tr>
<td>6.1</td>
</tr>
</tbody>
</table>

### Table 5

<table>
<thead>
<tr>
<th>Haptoglobin subtype distribution among matched pairs of Solomon Islanders dead by 1978 (Group D) and alive in 1978 (Group A)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haptoglobin subtype</strong></td>
</tr>
<tr>
<td>Group D</td>
</tr>
<tr>
<td>Group A</td>
</tr>
</tbody>
</table>

### Table 6

<table>
<thead>
<tr>
<th>Mean differences in serum transferrin and ferritin levels measured in 1966-1970 among matched pairs of Solomon Islanders dead by 1978 (Group D) and alive in 1978 (Group A) by year of death</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Year of death (Group D - Group A)</strong></td>
</tr>
<tr>
<td>1967-1970</td>
</tr>
<tr>
<td>1971-1973</td>
</tr>
<tr>
<td>1974-1977</td>
</tr>
<tr>
<td>1978-1980</td>
</tr>
</tbody>
</table>

A logistic model for matched pairs was applied to these data (26). We used the BMDPLR program (Biomedical Computer Programs, P-series, 1981, Health Sciences Computing Facility, University of California at Los Angeles) for logistic regression, which is oriented to the analysis of a prospective study design. By omitting the constant term, using the differences between Group D and Group A protein values, and assigning the outcome a success for all the matched pairs, this program can be used to estimate the coefficients (and odds ratios) associated with the explanatory variables of a conditional model (26).

There were 90 pairs with both ferritin and transferrin tested. As noted previously, matching was not entirely successful for all pairs. A subset of 76 pairs with complete matching was analyzed and the results were in accord with the results based on the full set of 90 pairs. These 90 pairs form the basis of the analyses that follow.
As Blumberg et al. (6) have noted, carriers of hepatitis B surface antigen maintain high levels of serum iron relative to noncarriers. Since ferritin and transferrin are related to serum iron levels, we included in the first model interaction terms of hepatitis B with each of these two proteins. In the second model, hemoglobin level was also included, as were Hp 0 phenotype and sex interaction terms with both ferritin and transferrin.

When four terms are fit (transferrin, ferritin, hepatitis B-ferritin interaction, and hepatitis B-transferrin interaction), only two make significant contributions to the likelihood: transferrin and the hepatitis B-ferritin interaction (table 7). The other two did not contribute to the fit to the data. The interaction term was generated by recoding hepatitis B status for each pair as 0 if noncarrier and 1 if carrier, then multiplying the ferritin (or transferrin) level of each member of the pair by the status.

A second model was applied which included hemoglobin level, haptoglobin variant (0 if Hp 0, and 1 if Hp 1-1, 1-2, or 2-2), sex-ferritin and sex-transferrin interaction, as well as the four variables used in the first model. In this model, transferrin and hepatitis B-ferritin interaction were retained in the best fitting model, and in addition, hemoglobin level was included. The estimated parameter values show that death is associated with low transferrin and low hemoglobin, and that among HBsAg carriers death is associated with high ferritin levels.

Estimated odds ratios are shown in table 8. The odds ratio approximates the risk of death in those exposed to the risk factors relative to those not exposed (relative risk). In this analysis exposure is continuous, not binary. The odds ratios shown compare the risk of death between an average member of Group D versus an average member of Group A; that is, between a person whose levels of serum pro-

### Table 7

Results of logistic regression analysis* applied to matched, retrospective study of the association of transferrin and ferritin levels with mortality in the Solomon Islands

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Standard error</th>
<th>Improvement chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>-0.0064</td>
<td>0.003</td>
</tr>
<tr>
<td>Ferritin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis B-transferrin interaction</td>
<td>0.025</td>
<td>0.013</td>
</tr>
<tr>
<td>Hepatitis B-ferritin interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>-0.007</td>
<td>0.0026</td>
</tr>
<tr>
<td>Ferritin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis B-transferrin interaction</td>
<td>0.022</td>
<td>0.012</td>
</tr>
<tr>
<td>Hepatitis B-ferritin interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-0.018</td>
<td>0.009</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex-ferritin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex-transferrin interaction</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* BMDPLR, Biomedical Computer Programs, P-series, 1981, Health Sciences Computing Facility, University of California at Los Angeles.
teins are the same as the averages in Group D and a person whose levels are the same as the averages in Group A. If more extreme values are chosen, the odds ratio becomes larger. For example, the results of this analysis suggest that the odds ratio (and approximately, the relative risk) is 3.0 for a hepatitis B carrier with a transferrin level of 225, ferritin level of 100, and hemoglobin of 11 compared with levels of 300, 75, and 13, respectively. Among noncarriers, the odds ratio would be 1.75.

We examined the mean ferritin differences between Group D and Group A by both sex and hepatitis B status. As can be seen in table 9, the ferritin differences were all positive and the transferrin differences were all negative in each hepatitis B status group among males. Among females, carriers and noncarriers of hepatitis B differed in the relationship of iron-binding proteins to mortality.

The results of fitting these logistic models accord with the interpretations drawn from the single-variable analysis: increased ferritin and decreased transferrin levels are associated with increased mortality, a finding consistent with the hypothesis that increased iron stores are associated with increased mortality. In addition, decreased hemoglobin level is associated with increased mortality.

**Table 8**

*Estimated odds ratios for carriers and noncarriers of hepatitis B, Solomon Islands study*

<table>
<thead>
<tr>
<th>Hepatitis B status</th>
<th>Carrier</th>
<th>Noncarrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Odds ratio</td>
<td>1.55</td>
<td>1.22</td>
</tr>
<tr>
<td>Upper 95% confidence limit</td>
<td>2.34</td>
<td>1.42</td>
</tr>
<tr>
<td>Lower 95% confidence limit</td>
<td>1.04</td>
<td>1.05</td>
</tr>
</tbody>
</table>

* Calculated from parameter estimates shown in table 7. Odds ratios compare a Solomon Islander with transferrin of 242, ferritin of 63, and hemoglobin of 12.4 (Group D (those dead by 1978) averages) against a Solomon Islander with values of 269, 52, and 13.1, respectively (Group A (those alive in 1978) averages).

**Discussion**

These data support the hypothesis that serum transferrin was decreased and serum ferritin was increased in 1966–1970 among Solomon Islanders who had died by 1978 compared with those who were still alive. Among females, the association of ferritin with mortality is greater in carriers of HBsAg than in noncarriers. To the extent that these proteins reflect available iron stores, our observations are consistent with our original hypothesis that iron stores were greater among those who were dead by 1978 than among a comparable group of age-, sex-, hepatitis B-, and village-matched alive controls.

There was about a 10 per cent difference between Group D and Group A in transferrin, and about a 20 per cent dif-

**Table 9**

*Mean differences in 1966–1970 serum ferritin and transferrin levels among matched pairs of Solomon Islanders dead by 1978 (Group D) and alive in 1978 (Group A) by sex and hepatitis B status*

<table>
<thead>
<tr>
<th>Hepatitis B status</th>
<th>Negative</th>
<th>Anti-HBs</th>
<th>HBsAg carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>Mean difference</td>
<td>(no.)</td>
<td>Mean difference</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>(25)</td>
<td>11</td>
</tr>
<tr>
<td>Female</td>
<td>-13</td>
<td>(24)</td>
<td>-18</td>
</tr>
<tr>
<td>Transferrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>-29</td>
<td>(25)</td>
<td>-24</td>
</tr>
<tr>
<td>Female</td>
<td>-25</td>
<td>(30)</td>
<td>-97</td>
</tr>
</tbody>
</table>
ference in ferritin. The manufacturers report a coefficient of variation of 3–8.5 per cent for the transferrin kit, and the manual reports a coefficient of variation of between 0.1–7.9 per cent for the ferritin procedure depending on the level in the serum. Our mean differences exceed these values; in addition, there is no reason to suspect that systematic measurement errors generated our observations.

Ferritin and transferrin levels are influenced by many factors and may not reflect iron stores alone (14, 27–30). However, in normal individuals ferritin can be an accurate indicator of available iron stores (9, 31, 32).

In a study of 40 children with kwashiorkor, McFarlane et al. (33) discuss the hazards of iron therapy in these patients. They found that the children who died had lower serum transferrin levels than the children who lived and inferred that higher available iron and consequent overwhelming infection contributed to the deaths. There was, however, no difference in serum albumin between the two groups. None of the Solomon Islanders we examined had serum albumin levels as low as the mean in the group of kwashiorkor victims described by McFarlane et al., and this is consistent with the notion that the albumin difference seen between Group D and Group A in our study probably does not reflect a nutritional difference accounting for differential survival. This is supported by the failure to find differences in height, weight, and skinfold measurements.

Neumann et al. (34) examined three groups of Ghanaian children with severe malnutrition, moderate undernutrition, and apparently adequate nutrition (control group). They found depressed albumin and transferrin and increased immunoglobulins in the severely malnourished children compared with the controls. Sixty per cent of severely malnourished subjects had hemoglobin below 11 g/dl. Eighteen per cent of the moderately undernourished children and 12.5 per cent of the controls had hemoglobin below this level. We found 19 per cent of Group D and 14 per cent of Group A with hemoglobin this low. The mean serum albumin levels of those in Group D who were under 20 years of age at the start of the study (11 children) was 39 g/liter, which was in the range of the control and moderately undernourished groups in the Neumann study. This supports the view that malnutrition was probably not an important factor in the Solomon Islands study group, and does not account for the differences in transferrin and ferritin levels which we found between Groups D and A.

Murray et al. (35) examined 137 iron-deficient Somali nomads, and alternately allocated nomads to receive 900 mg ferrous sulfate or placebo for 30 days. They observed that the iron-treated nomads had 36 infectious episodes while the placebo-treated had only six. The two groups were comparable in age, sex, and weight, and no participants were deemed to be undernourished or recovering from malnutrition. Iron deficiency was defined on the basis of hemoglobin, transferrin saturation, serum iron concentration, and a blood smear showing microcytic hypochromasia. They concluded that increased infection rates and reactivation of existing infection (tuberculosis, malaria, etc.) occurred after iron-deficient nomads reached what is considered normal iron levels.

In another study, Murray et al. (36) found that of two groups of Turkana tribesmen in Kenya, one eating milk and the other eating fish and milk, the fish eaters suffered more frequent infection. They stated that the only apparent difference between the groups was the lower serum iron of the milk drinkers. Among 230 milk drinkers, the mean serum iron was 38.2 µg/dl, while among 231 fish eaters, the mean iron was 59.7 µg/dl—both within normal bounds.

**Conclusion**

Decreased survival was found to be associated with elevated ferritin and de-
creased transferrin levels. This finding is consistent with the hypothesis that elevated iron stores increase the risk of death. This elevation could be due to a variety of factors operating in the Solomon Islands environment. Individuals with increased iron stores may be more prone to chronic and acute infection, and this may be reflected in elevated gamma globulin. The lowered hemoglobin could also be a consequence of chronic infection.

Among females, there is an interaction of the mortality-ferritin association with host response to hepatitis B infection; the relationship of ferritin to mortality is confined to the carrier group.

These results, if confirmed, would predict that a small annual relative risk associated with excess iron would lead to a significant number of deaths over a long time span. In our judgment, this study by itself should not be considered as sufficient evidence to change current nutritional practices. We hope that it will stimulate additional studies to retest the hypothesis.

REFERENCES


IRON AND IRON BINDING PROTEINS IN PERSISTENT GENERALISED LYMPHADENOPATHY AND AIDS

SIR,—Dr de Souza (Sept 17, p. 681) suggested investigating serum iron and ferritin levels and transferrin saturation in patients with acquired immunodeficiency syndrome (AIDS) to evaluate the possibility that iron overload is causally related to their immunodeficiency. We had already started such a study because of observations that increased serum ferritin levels before infection with hepatitis B virus were associated with the development of chronic hepatitis B infections, whereas low ferritin levels were associated with transient infections and development of antibody to hepatitis B surface antigen. Persistent generalised lymphadenopathy (PGL) is believed to be a prodrome of AIDS, and we thought that homosexual men with PGL in whom AIDS develops might have higher ferritin levels than PGL patients who do not proceed to AIDS.

We studied sera from 103 homosexual men (44 with AIDS, 55 with PGL, and 4 without PGL or AIDS). Ferritin was measured by radioimmunoassay ('RIANEN' kits; New England Nuclear), and serum iron was assayed by colorimetry. Serum ferritin levels were significantly higher (p<0.0001) in AIDS patients than in those with PGL (table). The ferritin values of the 4 homosexual men without AIDS or PGL were similar to those of the men with PGL. Serum iron concentrations were significantly decreased (p<0.0001) in AIDS patients compared with PGL patients. The iron levels in the PGL patients were lower than those in the 4 men without PGL or AIDS and lower than the iron concentrations usually found in normal adult males in the United States. Transferrin concentrations were not different in the AIDS and PGL populations, but the levels in the AIDS patients were lower than expected, given their relatively low iron concentrations.

<table>
<thead>
<tr>
<th>Serum iron (µg/dl)</th>
<th>Long serum ferritin* (ng/ml)</th>
<th>Serum transferrin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS (n=44)</td>
<td>PGL (n=55)</td>
<td>Neither (n=4)</td>
</tr>
<tr>
<td>82±8±5-1</td>
<td>2-6±20±0-070</td>
<td>293±7±14±5</td>
</tr>
<tr>
<td>116±6±2</td>
<td>1±9±0±4-7</td>
<td>340±3±17±3</td>
</tr>
<tr>
<td>140±8±6-0</td>
<td>2-06±0-153</td>
<td>240±3±13±6</td>
</tr>
</tbody>
</table>

*Log transformation of serum ferritin was used to normalise the skewed distribution.
Ferritin values ranged from 19±2 to 1070 ng/ml.

Thus serum ferritin levels seem to be raised and serum iron levels depressed in patients with AIDS. We had sera from 6 PGL patients in whom AIDS subsequently developed; the ferritin and iron concentrations in these sera were similar to the levels in sera from patients with clinically diagnosed AIDS. Rising levels of serum ferritin and falling concentrations of serum iron in PGL patients may herald progression to AIDS. This hypothesis will be tested in a controlled prospective study.

What is the possible explanation for the changes in iron and ferritin levels in the AIDS patients? Bacteria, parasites, and virus infected cells require iron for growth. Prokaryotes have siderophores which bind iron efficiently and compete with host binding proteins for iron. Serum ferritin probably originates largely from cells of the reticuloendothelial system, particularly fixed and circulating monocytes and macrophages. Patients with AIDS are probably carrying a heavy load of infectious agents which draw on body iron stores for their metabolism, and PGL patients who are progressing to AIDS may be in the process of acquiring large populations of microbial agents. The phagocytic cells of AIDS patients are actively ingesting these microbial agents, incorporating their iron and synthesising and releasing ferritin. If this is the correct explanation, it is likely that the phagocytic activity of these cells is also associated with the release of toxic oxygen radicals and lysosomal enzymes. These substances may damage lymphoid cells and thereby worsen the immunodeficiency of PGL and AIDS patients. Ferritin itself has immunosuppressive effects and the increased ferritin concentration may also contribute to immunodeficiency.

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The Daedalus Effect: Changes in Ethical Questions Relating to Hepatitis B Virus

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The Daedalus myth is a metaphor for aspects of the scientific process. When a problem is solved it often raises others, and when these in turn are solved they generate additional questions. Although perfect solutions may not be possible, major improvements can be made in many cases. Research on the hepatitis B virus is an example. The ability to detect carriers of hepatitis B virus contributed to the control of post-transfusion hepatitis but raised social and ethical problems inherent to the identification of carriers in the community. Partial solutions to this problem resulted from the ability to distinguish infectious from noninfectious carriers by the use of the hepatitis B e antigen (HBeAg) test and the development of an effective vaccine against hepatitis B virus. These solutions will undoubtedly lead to other problems and their solutions, which will in turn lead to other ethical and medical questions.

The Daedalus myth can be viewed as a metaphor for the problem-solving and problem-creating aspects of scientific life (1). Daedalus, a legendary figure of the classical world, was a craftsman, inventor, architect, and artist. He was also a generator and solver of problems. Every time a question was answered it raised several more, and these in turn led to other questions; his search was endless. Daedalus repeatedly learned that even the most ingenious solutions were neither flawless nor definitive. His actions had both positive and negative consequences for the persons affected. Some of these consequences were anticipated and others were not.

Daedalus must have been aware of the imperfect nature of his solutions, but he was captivated by the problems presented to him, encouraged by the progress that he made, and challenged by the new questions his solutions raised. He was also realistic and prudent and recognized limits beyond which he must not venture. Daedalus attempted to convey his perspectives on problem-solving to his son Icarus. But in keeping with the limitations and imperfections of which he was so keenly aware, he did not succeed in saving his son.

The medical scientist will recognize a familiar pattern in the career of Daedalus. When a hypothesis is tested it generates others, and when tested these yield still more. Scientific research has an infinite quality; the more we know, the more we know about what we do not know, and this unknown must in turn be understood. Each time a medical treatment, diagnostic technique, or public health measure is introduced, no matter how effective it may be, it nearly always raises other problems. All drugs have undesirable side effects; diagnostic procedures are rarely, if ever, entirely specific or infinitely sensitive; and public health measures often have medical, social, and even political consequences that are difficult to foresee.

The metaphor, however, is not pessimistic. It may be a paradox, but knowledge of what we do not know contributes to understanding and enlarges the imaginative possibilities of an investigation. There may be an infinite amount that can be known about a medical problem but it is amazing how little need be known in order to provide effective, although not perfect treatments. Penicillin, for example, provided remarkable treatment long before there was any understanding of its mode of action. Theavitaminoses were treated and prevented decades before there was any knowledge of vitamins, or even before they were named.

The solution of problems generates other problems; however, they are often of less consequence than the problems that were resolved. Drugs may have serious side effects, but the problems they solve, such as the cure of infectious disease by penicillin, are far more beneficial than the side effects that they cause. Anyone who remembers the era before antibiotic agents can testify to the immense improvement in patient care that resulted from the introduction of penicillin and the drugs that followed. Although perfection in medical care may not be possible, major improvement is, and the history of medicine in the past 50 years contains many examples of such advances.

The Daedalus metaphor is not only relevant to biomedical problem solving; it is also applicable to ethical questions associated with medicine. Medical research can raise ethical problems, but may also contribute to their resolution.

Daedalus

Daedalus, the legendary builder, designer, and inventor, murdered his nephew Perdix for reasons that are ambiguous. Daedalus fled Athens, went to Crete, and was employed at the court of King Minos. Known for his ability to make clever images of animals, Daedalus was employed by Pasaphae, the Queen of Crete and wife of Minos, when she became hopelessly enamoured of a great white bull presented to her and her husband as a gift by the god Poseidon (the mythic father of Minos). Daedalus designed and built a life-size, hollow cow. The queen positioned herself in the model, which was placed in a seductive situation in a field, and eventually she was impregnated by the white bull. Her problem, the need for
the bull, was solved, but it created another: the offspring of this unfortunate union was the half-beast, half-human Minotaur. This awful animal terrorized the citizens; the noise it created was like thunder, when it stamped its feet the earth shook, and it was a nuisance and danger in other ways as well.

Daedalus designed and built the labyrinth in which the Minotaur could be kept away from others. But the Minotaur required the sacrifice of young men and women. Each year, youths were sent from Athens to be offered up to his insatiable appetite. When Theseus, the son of the king of Athens, was sent with the sacrificial youths, Daedalus told him and Ariadne (the daughter of Minos and Pasaphae, thus half-sister of the Minotaur) of a secret door by which they could enter the labyrinth to slay the Minotaur. He also gave Ariadne a magic spool of thread that would unwind and show them how to escape the labyrinth after they had slain the beast. Theseus and Ariadne slew the Minotaur and fled Crete, with many adventures along the way. Ariadne was eventually abandoned by Theseus on the island of Naxos.

Hence, another problem was solved but others developed. Minos imprisoned Daedalus and his son Icarus in the labyrinth for their part in the death of the Minotaur and the escape of the killers; the mazemaker was trapped in his own maze—an ironic metaphor within a metaphor. But the imprisonment had its solution. Daedalus invented wings and fashioned them from feathers and wax. He and his son escaped their jailers but, in the ensuing flight, against paternal advice, Icarus flew too high. The heat of the sun melted the wax, the wings came away, and Icarus fell to his death in the sea that now bears his name. Daedalus flew on to the Greek colony of Cuma, south of Naples (or, in some versions of the myth, to Sicily), and additional adventures and travels followed.

The sun and its heat, the wings and their wax, the sea into which Icarus plummeted, his attitudes and his death, all became part of the continuing cycle of questions and answers, problems and solutions that are inherent to creative and responsible science and to human questing.

Bioethics and Medical Research

Bioethical problems cannot be fully understood without a thorough grasp of the scientific and technical phenomena that surround them. It is necessary to understand the scientific content of a problem in order to formulate the ethical problem. However, it is not the scientific factors alone that cause the ethical dilemma. There is always an interplay of belief, value, and social factors in ethical problems and their designation as such. Scientists and physicians need to understand this process in order to more knowledgeably and responsibly serve their patients and the general public. Advances in biological and medical knowledge do not only lead to ethical problems; they can also alleviate and even solve these problems. Continuing research often mitigates a previously difficult issue or changes it into one for which a solution can more readily be found.

Science is at once a problem-solving and problem-creating process. Each time a question is answered (a hypothesis effectively tested), it raises additional questions that in turn are answered and produce another generation of questions. The process may be infinite.

Another pattern in contemporary medical science ought to be considered in arriving at decisions as to when and how a scientific finding should be applied. Research is often adopted in clinical practice before it is actually completed. When the possibilities of a scientific advance are perceived, they are frequently applied to clinical practice as if their value had already been tested. Or, to put it in a more formal statement, a distinction is not made between the hypothesis when it is first stated ("Based on laboratory observation the hypothesis is made that drug Alpha is beneficial for cardiac arrythmias"), and the hypothesis after it has been adequately tested and supported ("We have already tested Alpha in a series of controlled trials and find it to be better than the previously available therapy"). In the early phase of research there often appears to be greater awareness and more frequent and enthusiastic reporting on the problem-solving ("beneficial") consequences of the discovery than on its problem-creating ("side effects") consequences. It is a common experience to read the glowing first accounts of a newly introduced procedure or therapy proclaiming its great effectiveness and nearly trouble-free character. Only later one learns that the procedure is not as effective as first thought and carries with it significant toxic or undesirable consequences.

These patterns are cultural as well as cognitive. The ritualized optimism that is involved and the questing hope that a "perfect" medical drug, procedure, or device can be found that has only good consequences and no bad ones are part of Western values and beliefs, especially in their American form (2). It is the first-hand experience of many, however, that a problem-free "magic bullet" form of scientific knowledge does not exist; perhaps it never will. Although commitment to the principle of constant improvement through new problems and their solutions is essential to science, it is unrealistic and in some respects unethical for scientific progress to be tied to a utopian and hubris-ridden notion of perfection. The practice of medicine cannot become perfect, but it can be made much better.

Bioethical Problems Related to the Detection of Hepatitis Carriers

The test for what was then known as the Australia antigen was reported in 1964 (3). By 1966, there was substantial evidence that Australia antigen was on the surface of hepatitis B virus. In 1967 (4), screening of blood donors for occult carriers of hepatitis virus was routine in Philadelphia, and by 1970 it was widely applied in the United States and other countries (4, 5).

In 1968, reports were heard from persons, usually health care personnel, who had been identified as carriers either as a result of a blood donor or hospital staff testing program. For example, a hospital nurse identified as a carrier was told that she would be fired because of the possibility that she might infect people by personal contact. An applicant for a hospital position was deemed
inhomosexual man was informed that he was a carrier. A homosexual man was informed that he was a carrier, but given no specific instructions on how he should conduct himself socially.

Along with the many incidents of this sort, policy questions about hepatitis B virus carriers were raised by various institutions. Military medical authorities in a foreign country where hepatitis B virus carriers were common asked if they should screen applicants for admission to medical school and disqualify those found to be carriers. The same question was raised with respect to admission to officers' training school and graduation from nursing school: should these applicants and graduates be screened for the carrier state to determine if they should be allowed to enter, or undertake the practice of their chosen professions? (We recommended that screening should not be done.) Still another difficult policy problem arose in relation to the adoption of Southeast Asian refugee children. In the mid-1970s, many children from Indochina (primarily orphans, but in some cases children who still had parents) were placed for adoption. Because hepatitis B virus carriers were known to be prevalent in Vietnam, the advisability of screening these children for the virus became an issue. Should the results of this screening test determine who would be accepted for adoption and who would not? (United States health officials decided that carrier testing should not be done.) These carrier-related questions surfaced in increasing numbers as the use of the hepatitis B virus test became more widespread.

What appeared to be emerging was the possibility that a new class of stigmatized persons and groups—hepatitis B virus carriers—was being created by the introduction of a single laboratory test (6). These were persons who did not have any recognizable external characteristics. Their carrier state was “occult,” only discernible by use of a blood test that had been introduced. Many carriers already had been, and even more would be, identified as a result of the donor and other blood-testing programs that had been launched. (Tens of millions of donor blood samples are collected each year, and in due course most of these would be tested.)

It was known that some carriers could transmit hepatitis readily by blood transfusion. From this knowledge, but without quantitative data on actual transmission, it was inferred that carriers could also convey hepatitis through social interaction. This assumption began to take hold despite the fact that it was apparent that most carriers were not very infectious. It had been estimated that there were about 700,000 carriers throughout the United States. If they were very infectious—for example, to the degree that people infected with smallpox are—there would have been far more hepatitis in this country and elsewhere than there is known to be. Furthermore, several preliminary studies of health care workers known to be carriers showed that they had not transmitted hepatitis B virus to their patients. The scientific evidence strongly suggested that, although some carriers might be infectious, many were much less so if at all, and that the danger to public health was probably not immediate or enormous. Nevertheless, persons who had been identified as hepatitis B virus carriers were being medically and socially marked in potentially disadvantageous ways. They were having employment and career difficulties as well as interpersonal and family embarrassment and stress as a result of their disclosed carrier status.

Consideration of these problems made it clear that an evaluation of a general screening program should be done before any such program was organized. (This program refers to the screening of persons other than blood donors. Blood donor screening had been evaluated, found to be justified, and accepted.) What was called for was a judicious, well-informed set of decisions about screening that would potentiate its public health benefits, and protect individual hepatitis carriers from undue economic, psychic, and social harm. It became apparent that there were not sufficient data on which to make sound decisions that would appropriately balance collective needs and individual rights. It was not at all clear what the rules for a screening program ought to be. Should screening be compulsory? Should the activities of carriers be restricted and, if so, in what way? How could infectious carriers be distinguished from those who were not infectious? What instructions should be given to identified carriers? What kind of protection could and should be offered to those with whom carriers came in contact? The history of previous medical applications strongly suggested that these issues should be addressed before the screening procedure acquired the routine familiarity and authority of an established practice; once a procedure has been instituted, it becomes increasingly hard to question or freshly evaluate it.

There were also broader issues that had been raised and required consideration. Most of the infectious diseases that people contract are transmitted either directly or indirectly from other people, in many cases from carriers. However, screening for these agents is difficult and not done routinely on large segments of the general population. For example, Staphylococcus aureus carried on skin surfaces may be spread from person to person and has caused large and calamitous epidemics in hospital nurseries. Nevertheless, routine screening is not done for this bacteria except after infection has been found. Salmonella species may be spread by food handlers and cause serious epidemics of diarrheal disease, but routine screening is not done because of its expense and difficulty. Should hepatitis carriers be targeted for screening simply because the test is easy to do and widely available? Beyond this, how much should biological knowledge be allowed to influence and control our social relationships? To what extent should medical and public health practices be allowed to affect our social behavior—particularly in the face of the kind of inadequate information about hepatitis B virus carriers and the infectious risk they constitute that existed at this point?

For various reasons, hepatitis screening for blood donors was accepted quickly, whereas screening for many other infectious agents has not been accepted. There was an unambiguous, long-standing need to screen blood donors for hepatitis. Post-transfusion hepatitis was a real
and significant problem that had been recognized for years and any solution was bound to be accepted quickly. The tests for hepatitis B virus (particularly the radioimmunoassay that was introduced relatively early in the program) were sensitive and specific. There was a large commercial interest in these tests. Test reagents for hepatitis B virus to the value of tens of millions of dollars are sold yearly, aided by extensive advertising and skilled promotion. Further, several law suits had been brought against hospitals, blood banks, and physicians by defendants who developed post-transfusion hepatitis and claimed that the institution and health care workers were liable because they had not used the screening test for hepatitis B virus. In addition, blood has a powerful symbolic meaning in our culture. It is associated with life and vigor, lineage and kinship, and sacrifice and the sacred in ways that are likely to confer special positive significance on technical procedures that guarantee its “purity.”

The question of a general screening program (the testing of persons who were not blood donors) had to be viewed very differently. We, and others in the field, took the position that there had not yet been enough research on hepatitis B virus carriers to justify general screening programs, except for those that were part of a research protocol (7). Adequate research had been done to support the screening of blood donors, and here the balance was clearly in favor of the public health. But the research was not yet sufficient to know how to use the results of a general screening program. Continued research to resolve the social and ethical as well as scientific and medical aspects of these problems was recommended. Concrete projects for research, several of which were already in progress, were also advocated (7).

Advances in Research and Their Impact on Ethical Questions

Since our original publication on these issues, there have been scientific and technical advances that have changed the ethical issues in hepatitis B virus carrier screening, and altered our views in the process. In 1972, Magnus and Espmark (8) reported their finding of the hepatitis B e antigen (HBeAg). This antigen appears to be a part of the core of hepatitis B virus, and its presence in a carrier indicates that significant amounts of whole infectious virus are present in the blood (9, 10). Several studies have shown that carriers of hepatitis B surface antigen (HBsAg) who also have HBeAg are much more likely to be infectious than carriers without HBeAg, as seen in transmissions from a carrier mother to her offspring, and in horizontal transmission from one person to another. Furthermore, HBsAg carriers who also have antibody to hepatitis B e antigen (anti-HBe) in their blood are much less likely to transmit the agent than those with HBeAg or those without any sign of HBe antigen or antibody. By separating the carrier group into those who are potentially infectious and those unlikely to transmit hepatitis, the problems of screening were narrowed and focused.

In 1969, Millman and Blumberg introduced a unique method for the production of a vaccine to prevent infection with hepatitis B virus (11). The vaccine has been tested (12), accepted by government agencies, and is now widely used by individuals and for public health projects in this country and abroad. If the use of the vaccine continues to be as safe and effective as these original trials indicate, it will be possible to protect persons with whom carriers come in contact. In time, in some regions of the world where hepatitis prevalence is high, all or nearly all of the population will either have natural protection against hepatitis B virus (they will have developed the antibody to hepatitis B surface antigen [anti-HBs] after natural infection with the virus), or they will have been vaccinated. When this happens, the public health impact of the carriers will be greatly minimized in these areas. As a consequence, the chief ethical problem of the carrier will have been eliminated.

In areas with low intensity of hepatitis B virus infection, the vaccine will probably only be used in high-risk populations, including health care personnel, travelers, military personnel, blood handlers, homosexual men (who have a high risk of infection with hepatitis B virus), drug abusers, family members of carriers, and certain other groups. In some of these high-exposure populations, the frequency of naturally occurring anti-HBs may be common (15% to 50%). Because the cost of vaccine is high, it would be prudent in some areas to screen these populations for the presence of anti-HBs (and possibly HBsAg), because they would not profit from vaccination. Under these changed circumstances, the screening of general populations would be warranted. The initial concerns about general population surveys would be set aside, because measures of known value could be taken as a consequence of the survey.

There are other situations in which screening surveys may be warranted. Current policy encourages the “mainstreaming” of mentally retarded children by placing them in small, home-like settings in the general community, and in regular schools with other children. There is a relatively high frequency of carriers among children and young adults with Down’s syndrome, who make up a sizeable portion of deinstitutionalized, mentally retarded people. Research should continue to determine the risk that these children may impose on their classmates, and on the feasibility of vaccine protection. Similarly, adopted children from communities with high frequencies of carriers (for example, Southeast Asians) may also be screened after adoption has occurred. Vaccine could then be recommended to family members if the adopted child is found to be a carrier and have HBeAg. In selected cases, consensual partners may request screening tests in order to decide if an uninfected partner should be vaccinated.

Nevertheless, these screening procedures will continue to raise ethical problems. Although there is now a more obvious public health value that would derive from screening in certain populations, the identification of hepatitis carriers still involves some degree of individual and group stigmatization and is a potential disadvantage. A recent example illustrates the difficult problems that may develop. A limited screening program of East Asian immigrants living in the United States was part of a vac-
cination project. A young student about to attend a private, residential school, was found to be a carrier. This raised the question of whether the school should be informed, and, if so, in what way? Should the student's roommate be vaccinated? Should other students be vaccinated? Who should pay for these vaccinations? Would knowledge of the fact that this student was a carrier adversely affect the way he was viewed by other students? What repercussions might this have for attitudes toward the student's ethnic group? This last possibility was of special concern to the immigrants community who had come to the United States from an area of Asia where hepatitis B virus is endemic. Not only was the community worried about the prejudice and discrimination that the student might experience, but also about the "impure" image of their ethnic group that might emerge if a significant number of carriers were identified in their community. Would Americans come to regard their whole group as "marked" by hepatitis, and, consequent­ly, as a public threat? What implications would this have for the respect and status they would be accorded in American society, and for their educational, occupational, and economic opportunities for advancement and success? In this particular case, such questions (some of which were explicitly stated, others of which were im­plied) became so acutely problematic that government health authorities were consulted. In the end, the issues surrounding the case were only partly resolved.

Other advances that would further alter the ethics of screening are likely to occur. Research is now being directed toward an understanding of how to either eliminate hepatitis B virus from carriers, or to effect virus replication so that the amount of virus produced and excreted is so low that the carrier is no longer an infectious hazard (13). If such measures become possible, they would probably also decrease the likelihood of the carrier developing chronic liver disease or primary cancer of the liver. Under these conditions, identified carriers could be offered a quasi-therapeutic procedure. (It is not exactly therapy because carriers often do not perceive any illness.) This would provide a powerful new medical and moral rationale for general hepatitis B virus carrier screening that might further offset the negative personal, social, and cultural side effects associated with it.

Summary

We have used the term "Daedalus effect" to identify and describe the never-perfect nature of medical research and practice: the way in which each solution to a problem generates new problems. We have shown that this effect can relate to bioethical as well as biomedical problems.

It may be important for scientists and practitioners to recognize this phenomenon. It can help them to distinguish between the imperfections that are a consequence of the "Daedalus effect," and those that emanate from inadequate scientific thought or poor experimental design. Comprehension of this effect may help to mitigate both the hubris that results from the expectation that perfect solutions are possible, and the discouragement that comes from not achieving all desired ends.

Although it may not be possible to achieve perfection, it is within the capacity of research to make things much better than they currently are. For the contemporary medical investigator and practitioner this is a goal, as it was for Daedalus.

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To the Editor: Ted Slavin died on the 15th of November, 1984, from the ravages of hemophilia, kidney disease, and congestive heart failure. His death was a blow to all of us in the Division of Clinical Research of the Fox Chase Cancer Center.

Mr. Slavin contracted hepatitis B in the mid-1950s, and probably as a consequence of subsequent repeated exposure to hepatitis B virus in numerous transfusions (blood donors were not screened for the virus at that time), he had very high titers of antibodies against hepatitis B. In 1970 the Hemophilia Society began to test patients for hepatitis B viral markers, and the high titers of antibodies in his blood were revealed. Slavin realized the value of his blood both to commercial organizations that at the time were using human serum in manufacturing diagnostic kits for hepatitis B virus, and to laboratories attempting to advance knowledge about blood-borne human viruses. Ironically, Slavin's blood, which previously had been an enormous burden to him, then became an asset. He began to market his blood, on occasion for up to $10.00 a milliliter, and later organized a company, Essential Biologicals (a particularly appropriate name), to collect blood from persons with rare or unique blood factors. Through his energy and wit, he converted a disastrous health condition into a successful business.

In addition to its commercial use, Slavin provided his serum, at no cost, for research purposes. From the National Institutes of Health he obtained a printout of workers in the hepatitis field and selected our laboratory as a recipient of his blood. We used it in research on the radioimmunoassay test, tissue fluorescence techniques, the development of the vaccine against hepatitis B virus, and the prevention of primary cancer of the liver.

We will long remember Ted Slavin as a gallant man who loved life and who contributed greatly to our research efforts.

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