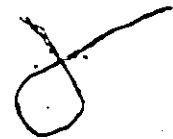


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LA THÈSE A ÉTÉ
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IMMUNOLOGICAL STUDIES IN HEPATITIS B

A Thesis submitted to the
Faculty of Graduate Studies
University of Ottawa

In Partial Fulfillment of
the Requirements for the Degree
Doctor of Philosophy

by

Morris Jutcovich

September, 1980

Dedicated to my Mother and Father

whose love made this possible

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ACKNOWLEDGEMENTS

I am deeply indebted to Dr. Edgar Perry for his assistance, advice, patience and above all, friendship; without these, this project could never have been completed, let alone attempted. I would also like to thank Dr. J.C.N. Westwood, Chairman of the Department of Microbiology and Immunology for his timely advice and for the opportunity to work in the department and Dr. E. Rossier for his advice and discussions on cell mediated immunity. In addition, I would like to thank the other staff members of the Department of Microbiology and Immunology whose acceptance made my stay so much more enjoyable.

I would also like to thank Dr. J.L. Gerin, Director of the MAN Program, Rockville, Maryland, Dr. R.H. Purcell, NIAID, Bethesda, Maryland, Dr. E. Overby, Abbott Labs., N. Chicago, Ill., Drs. R.Y. Dodd, and N. Nath, American Red Cross, Bethesda, Maryland and Dr. S.Z. Hirschman, Mount Sinai School of Medicine, New York City for allowing me to work in their laboratories and for teaching me invaluable technical skills needed for this project.

Last, but not least, I would like to thank the support staff in the Department of Microbiology and Immunology for their assistance - Mr. A. Brunner for assistance with electron microscopy, Mr. C.J. McCulloch for technical assistance, Mr. P. Gay for always making sure I had the right supplies when needed, Mrs. H. Baraniak and Mrs. K. Laschewski for supplying me with clean glassware at all times, Mr. W. Brennan and the Animal House staff, Mrs. Terry Goldberger and Monique D'Amour for their efficient typing, and to all other staff whose assistance from time to time was gratefully appreciated.

I have been fortunate to work with such a fine group of people. It certainly made my job a lot easier.

SUMMARY

1. Three assays were compared for their ability to detect HB_SAg in serum. RIA was found to be more sensitive than CIEP and AGD. We also compared RIA and HA for detecting anti-HB_S and found them to be equally sensitive.
2. Subtypes of four HB_SAg-positive populations were determined. Residents of mental institutions and renal dialysis patients were predominantly HB_SAg/ad. The majority of blood donors also had this subtype while acutely ill patients were predominantly HB_SAg/ay positive.
3. Two HB_SAg purification schemes are presented. Both involve three isopycnic bandings in CsCl and a rate separation step in a continuous sucrose gradient. The final purified antigen particles have a diameter of 20 nm, a buoyant density of 1.20 g/cm³ in CsCl and is free of detectable human serum contaminants by AGD; occasionally these preparations raise anti-HSA antibodies when injected into guinea pigs.
4. HB_CAg was purified from the liver and serum. The core particles from the liver were "empty", banded at 1.30 g/cm³ in CsCl and were negative for DNA polymerase. Two populations of cores were extracted from serum Dane

- particles - an "empty" population which was identical to the particles obtained from hepatic tissue and a population of "full" particles which banded at 1.36 g/cm^3 in CsCl and was positive for DNA polymerase activity. Both populations had diameters of 27 nm.
5. Four HB_sAg-positive populations were tested for HB_eAg. This antigen was most frequently found in the renal dialysis population and least frequently encountered in blood donors with intermediate frequencies for the DS and OMR residents of mental institutions. Prevalence of HB_eAg was dependent on the length of residence rather than the age at admission for these two latter groups.
 6. HB_eAg was more often detected in HB_sAg/ay positive blood donors while anti-HB_e was more often found in the HB_sAg/ad positive donors.
 7. A third e marker was detected and designated as e₃. It was similar to e₂ in that it appeared and disappeared at the same time as this determinant and was thermolabile at 56°C. A more appropriate designation may be e_{2a}.
 8. HBV specific DNA polymerase activity was detected in 80% of HB_eAg-positive sera and in 36% of the HB_eAg/anti-HB_e negative sera. This latter finding is thought to be due to the relative insensitivity of the AGD assay in detecting HB_eAg-positive sera.
 9. All HB_eAg/DNA polymerase positive sera contain full and empty Dane particles which can be visualized by EM.

An occasional anti-HB_e positive serum also has detectable Dane particles; however, their numbers are reduced and all are empty.

10. Lymphocytes from 16 post-hepatitis B patients challenged in vitro with purified HB_sAg and the inactivated NIH hepatitis B vaccine showed no significant transformation. A contaminant fraction obtained during the purification process was found to significantly stimulate lymphocytes from this population. These findings may explain some of the earlier positive CMI responses obtained by some investigators using partially-purified HB_sAg preparations.
11. The leukocyte migration inhibition test was performed using the agarose method described by Clausen (1971). No significant inhibition of the migrating polymorphonuclear leukocytes was observed, confirming the results of some investigators and refuting those of others.

ABBREVIATIONS USED

AGD	Agarose gel diffusion
anti-HB _c	antibody to hepatitis B core antigen
anti-HB _e	antibody to hepatitis B e antigen
anti-HB _s	antibody to hepatitis B surface antigen
anti-NHS	antibody to normal human serum
ARC	American Red Cross
B-cell	Bone-marrow derived lymphocyte
CaCl ₂	Calcium chloride
CsCl	Cesium chloride
CMI	Cell mediated immunity
CIEP	Counter immunoelectrophoresis
CPM	Counts per minute
cm	centimetre
cc	cubic centimetre
Ci	curie
CS1	1st isopycnic banding product
CS2	2nd isopycnic banding product
CS2R1	Rate separation product
CS2R1CS1	3rd isopycnic banding product
d ATP	deoxyadenosine-5' triphosphate
d CTP	deoxycytidine-5' triphosphate
d GTP	deoxyguanosine-5' triphosphate
DHT	Delayed hypersensitivity testing

DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DPA	DNA polymerase assay
DPSA	DNA polymerase specificity assay
DS	Down's syndrome
DTH	Delayed type hypersensitivity
EM	Electron microscopy
FBS	Fetal bovine serum
g	gram
g/cm ³	gram per cubic centimetre (a measure of density)
HA	Passive hemagglutination
HAA	Hepatitis-associated antigen
HB _c Ag	Hepatitis B core antigen
HB _e Ag	Hepatitis B e antigen
HB _s Ag	Hepatitis B surface antigen
HBIG	Hepatitis B immune globulin
HBSS	Hanks balanced salt solution
HSA	Human serum albumin
³ H-T	Tritiated thymidine
³ H-TTP	5 ³ H-thymidine-5' triphosphate
IEM	Immune electron microscopy
K cell	Killer cell
KCl	Potassium chloride
L	Litre
LSM	Lymphocyte separation medium
LT	Lymphocyte transformation
M	Molar
MgCl ₂	Magnesium chloride

MI	Migration index
MIF	Migration inhibition factor
ml	millilitre
mm	millimetre
mw	molecular weight
N	Normal
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH ₄ Cl	Ammonium chloride
nm	nanometre
NP40	Nonidet P40
Ø	Diameter
OMR	Other mentally retarded
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PHA	Phytohaemagglutinin
P/N	Positive to negative ratio
psi	pounds per square inch
PTA	Phosphotungstic acid
RD	Renal dialysis
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Rowell Park Memorial Institute
s	Sedimentation coefficient
SDS	Sodium dodecyl sulphate
SGOT	Serum glutamic oxalacetic transaminase

SGPT	Serum glutamic pyruvic transaminase
SI	Stimulation index
SPP	Sodium pyrophosphate
TC 199	Tissue culture medium 199
TCA	Trichloroacetic acid
T-cell	Thymus-derived lymphocyte
T _G or T _γ cells	T lymphocyte with a surface receptor for the Fc fragment of IgG; suppressed cell
T _M or T _μ cells	T lymphocyte with a surface receptor for the Fc fragment of IgM; helper cell
μ	Micron
μCi	Microcurie
μg	Microgram
μl	Microlitre
UV	Ultraviolet
w/w	Weight to Weight

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INTRODUCTION

Viral hepatitis is a major public health problem in Canada and the United States not only in terms of its overall morbidity but also of its economic impact and demands on medical resources. Of all diseases currently reported to the Center for Disease Control (CDC) in Atlanta, it ranks behind only gonorrhoea, chickenpox and mumps in the number of cases reported annually: 56,623 in 1977, a rate of 26.2 per 100,000 population (VHSA, 1979). The combination of underreporting--Mosley and Galambos (1969) estimated that only between 10% and 20% of all cases are reported annually--and clinically inapparent infections suggests that the true incidence of this disease is grossly underestimated. The economic impact in terms of direct costs (diagnosis, treatment and prevention) and indirect costs (productivity losses due to restricted activity and premature death) is substantial. In 1970, these costs were estimated to be in excess of \$650 million annually (Tolsma and Bryan, 1976), and even though the annual rate of the disease has decreased from 32.0 per 100,000 population in 1970 to 26.2 in 1977, costs now are probably well in excess of \$1 billion per year.

Viral hepatitis was, until, very recently, thought to consist of two distinct disease entities, based on epidemiologic observations--routes of infection and periods of incubation--and on the results of studies of transmission in human volunteers. Numerous terms were used to describe these two forms of hepatitis, as can be seen in Table 1 from the World Health Organization (WHO, 1973), often leading to confusion amongst researchers and clinicians alike. A simple nomenclature had been suggested by MacCallum (1948) using the terms hepatitis A and hepatitis B to designate infectious or short incubation hepatitis and serum or long incubation hepatitis respectively. It was only following a recommendation by the WHO Scientific Committee on Viral Hepatitis (1973) that the terms hepatitis A and hepatitis B began to be used on a world-wide scale. Shortly after this report, a third type of hepatitis was described, differing from the other two etiologically, epidemiologically and immunologically and was called unspecified or non A, non B hepatitis (Feinstone et al, 1975).

I. Literature Review

Many of our current concepts of viral hepatitis are the results of studies on human volunteers conducted during the early 1940's (Voegt, 1942; MacCallum and Bradley, 1944; Havens et al, 1944; Neefe et al, 1945).

TABLE I

SYNONYMS FOR VIRAL HEPATITIS

Hepatitis A	Hepatitis B
Acute catarrhal jaundice	Arsenotherapy hepatitis
Acute viral hepatitis	Au (1)-hepatitis
A-LH hepatitis	Au/SH hepatitis
Australia antigen-negative hepatitis	Australia antigen hepatitis
Botkin's disease	B-SH hepatitis
Catarrhal jaundice	Hepatitis B (H.B.)
Common infective hepatic jaundice	Hippy hepatitis
Common-source hepatitis	Homologous serum hepatitis (Virus B)
Epidemic catarrhal jaundice	Homologous serum jaundice (HSJ)
Epidemic hepatitis	Inoculation hepatitis
Epidemic jaundice	Long-incubation hepatitis
Hepatitis A (H.A.)	MS-2 hepatitis
Icterus epidemicus	Parenteral hepatitis
Infectious hepatitis	Post-arsphenamine jaundice
Infectious jaundice	Post-transfusion hepatitis
Infective hepatitis (Virus A)	Post-vaccinal jaundice
Jaunisse des camps	Salvarsan jaundice
MS-1 hepatitis	Serum (MS-2) hepatitis
Short-incubation hepatitis	Serum hepatitis (SH)
Soldatengelbsucht	Serum jaundice
Viral hepatitis type A	Syringe jaundice
	Syringe-transmitted jaundice
	Tattoo jaundice
	Transfusion-associated hepatitis
	Transfusion hepatitis
	Viral hepatitis type B
	Yellow fever vaccine hepatitis

SOURCE: World Health Organization Technical Report, Series 512, 1973.

From their work, it was evident that two distinct forms of the disease existed. One was primarily an enteric infection, characterized by a relatively short incubation period, and with fecal excretion of the infectious material. This type was designated infectious hepatitis, or hepatitis A. Serum hepatitis, or hepatitis B, on the other hand was transmissible only by the parenteral route, had a relatively long incubation period and had no fecal excretion of the virus.

Historically, it is probable that hepatitis A is synonymous with the epidemic jaundice described many hundreds of years ago by Hippocrates. This disease was also described by Pope Zacharias in the eighth century A.D. who noted that its spread could be prevented if jaundiced patients were separated from the rest of the population. Outbreaks of this disease were particularly common during war years when hygienic standards were often low, with resultant contamination of food and water supplies. During World War II, it was estimated that approximately 5 million German soldiers and civilians had this disease (Carver and Seto, 1974).

The history of hepatitis B, on the other hand, is a more recent one. The first reported outbreak occurred in 1883 when 191 of 1289 Bremen ship-yard workers developed hepatitis after vaccination against smallpox using human lymph material (Lürman, 1885). Further hepatitis B outbreaks were documented in 1909 among patients at V.D.

clinics treated with salvarsan I.V. therapy. The best documented outbreak, however, occurred in 1942 when 28,585 American soldiers, inoculated with yellow fever vaccine "stabilized with human serum" developed jaundice; 62 died. In retrospect, it became obvious that the human serum component of the vaccine was contaminated with hepatitis B.

Sporadic reports of non-parenterally transmitted hepatitis B appeared in the literature (Propert, 1938; Findlay et al, 1944; Bradley, 1946; Neefe et al, 1946; Mirick and Shank, 1959), but most clinicians either did not accept these findings or tended to ignore them. Based on the human volunteer studies, the overwhelming view remained that hepatitis B was parenterally transmitted and was, therefore, not transmissible by other routes. This view, however, began to change somewhat in 1967 when Krugman et al, working at the Willowbrook School on Staten Island, New York, an institution for the mentally retarded, described two distinct forms of the disease based on clinical, immunological and epidemiological characteristics. These were designated MS-1 and MS-2. Type MS-1, resembling hepatitis A, produced hepatitis 35 to 57 days after the injection or ingestion of filtrates of infectious serum or feces. Serum transaminase levels were elevated from 3 to 19 days, thymol turbidity was consistently abnormal and the disease was highly contagious. MS-2, on the other hand, was similar to hepatitis B, producing the disease 41 to 108 days after injection or 70 to 130 days after

ingestion of infected material. The oral route, however, required a 50-fold increase in the dose to be infective. Transaminase levels were elevated from 35 to 200 days, thymol turbidity was usually within normal limits, and the disease was considered moderately contagious. These findings, and those of Hersh et al (1971) who also reported non-parenteral transmission of long-incubation hepatitis, have raised numerous questions as to whether earlier epidemiologic studies, distinguishing these two forms of the disease primarily by mode of transmission and length of incubation, are at all valid. Nevertheless, it was appreciated that two distinct diseases did indeed exist and it would be advantageous if some marker were available to distinguish between the two.

In 1961 Allison and Blumberg described precipitating antibodies in the sera of people who had received multiple transfusions. Additional studies revealed that patients with thalassemia who had received multiple transfusions developed precipitating antibodies against low density beta lipoproteins. A systematic study of these low density lipoproteins found an abnormal serum lipoprotein, very rare in the general population, that contained far less lipid than normal serum beta lipoproteins (Blumberg et al, 1962). This unique antigen was identified when a panel of 24 sera were tested in a two dimensional Ouchterlony immunodiffusion test against the sera of two hemophiliac patients who had received multiple transfusions.

The only serum that reacted was from an Australian aborigine and it was appropriately named the "Australia antigen" (Blumberg, 1964). The next task was to determine the worldwide prevalence of this antigen. It was found to be relatively rare in North America and Europe but significantly more prevalent in the tropical and South-east Asian populations (Blumberg et al, 1965). An interesting finding of this study was the relatively high frequency of this antigen in the sera of acute leukemic patients, an incidence of 11.4% in 70 patients tested. This led the group to speculate that:

(1) persons with Australia antigen have an increased susceptibility to 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.

Studies in 1967 and 1968 confirmed earlier findings by Blumberg et al that the Australia antigen was far more prevalent in tropical inhabitants. In addition to its high frequency in leukemic patients, it was also common in patients with Hodgkin's disease, lepromatous leprosy and viral hepatitis and in institutionalized Down's syndrome patients (Blumberg et al, 1968). The association with viral hepatitis was confirmed by other investigators (Okochi and Murakami, 1968; Prince, 1968) but some question remained as to whether it was a marker for one or both of the viral

hepatitis diseases. Initial reports associated this new antigen with both hepatitis A and B (Blumberg et al, 1968; Okochi and Murakami, 1968; Gocke and Kavey, 1969) but reports by other investigators (Giles et al, 1969; Krugman and Giles, 1970; Barker et al, 1970) confirmed earlier studies by Prince (1968) that the Australia antigen, or hepatitis associated antigen (Prince, 1968) was specifically a hepatitis B marker. Giles et al (1969) detected the antigen in 39 of 40 cases of MS-2 hepatitis and in none of 41 consecutive cases of MS-1, confirming that MS-2 hepatitis was, in reality, hepatitis B.

With the discovery of a specific antigenic marker for hepatitis B came improvements in the techniques for its detection. These included, in ascending order of sensitivity, the original agar gel diffusion described by Blumberg (1964), counterimmunoelectrophoresis (Shulman, 1970; Gocke and Howe, 1970; Prince and Burke, 1970; Alter et al, 1971; Mayumi et al, 1971), complement fixation (Gerin et al, 1969; Shulman, 1970; Rightsel and Muirhead, 1971; Kelen et al, 1971; Alter et al, 1971; Schmidt and Lennette, 1970; Schmidt et al, 1971), immunofluorescence (Coyne et al, 1970), electron microscopy (Shulman, 1970; Kelen et al, 1971), hemagglutination and hemagglutination inhibition (Vyas and Shulman, 1970; Mayumi et al, 1971) and radioimmunoassay (Walsh et al, 1970; Lander et al, 1971; Hollinger et al, 1971). Detection of hepatitis B antigen by these various techniques has dramatically helped

to decrease the incidence of post transfusion hepatitis. Prior to screening for this antigen, it was estimated that blood transfusions resulted in 30,000 cases of icteric hepatitis in the United States annually, 150,000 cases of anicteric hepatitis and 3,000 deaths (Krugman, 1971).

This antigen was detectable not only in serum but also in the feces of patients with hepatitis B (Grob and Jemelka 1971), urine (Skinhøj and Steiness, 1972; Apostolov et al, 1971; Blainey et al, 1971), saliva and mouth washings (Ward et al, 1972), semen (Heathcote et al, 1974) and nasopharyngeal washings and menstrual fluid (Melnick et al, 1976). Many elaborate studies were also undertaken to examine the possibility of venereal mode of hepatitis B transmission in heterosexual and homosexual populations (Jeffries et al, 1973; Fulford et al, 1973; Henigst, 1973; Szmuness et al, 1976). Evidence for this mode of transmission was highly suggestive in the homosexual population and less so in the heterosexual group, but in no study was it conclusive.

Many investigators next turned their attention to the morphology, biophysical and biochemical characteristics and specificity of this antigen. Bayer et al (1968) partially purified hepatitis B positive serum on sucrose density gradients and was able to see small spherical particles and long filaments by electron microscopy. These findings were confirmed by other investigators (Gerin et al, 1969; Millman et al, 1969; Almeida et al, 1969; Barker et al, 1969). The small spherical particles had

diameters of approximately 22nm, roughly the size that had been suggested for the hepatitis virus in earlier studies (McCollum, 1952). Diameters, however, varied with some groups of researchers, ranging from 16 to 25nm in one (Almeida et al, 1969) and 13 to 38nm in another (Kim and Tilles, 1973). The diameters of the filaments were comparable to those of the 22nm spherical particles, but their lengths extended to several hundred nanometers.

In 1970, Dane et al described a double layered, 42nm diameter structure with a 28nm electron dense inner core in hepatitis B positive sera. This particle was called the Dane particle and it was suggested that it represented the hepatitis B virion. This finding was confirmed by other researchers (Cossart and Field, 1970; Gust et al, 1970; Almeida et al, 1971).

It soon became apparent that the 22nm spherical particles, long filaments and Dane particles shared a common surface antigen determinant, referred to as hepatitis B surface antigen or HB_sAg (see Appendix I). Almeida (1971) showed that precipitation lines formed by HB_sAg and its antibody, anti-HB_s cut out from agar gel were composed of 22nm particles. In addition, when anti-HB_s positive serum was combined with serum containing HB_sAg, aggregates containing 22nm particles (Bayer et al, 1968; Almeida et al, 1969; Hirschman et al, 1969), long filaments (Almeida et al, 1969; Almeida, 1971; Gerin, 1972) and Dane particles (Dane et al, 1970; Almeida et al, 1971; Almeida, 1971;

Gerin, 1972) were specifically formed.

The relative numbers of these three particles in HB_sAg positive serum differed from serum to serum (Gerin, 1972; Almeida, 1972). Bond and Hall (1972) determined particle concentrations following a single isopycnic banding in cesium chloride (CsCl) and found the relative numbers of 22nm particles, filaments and Dane particles to be 1730, 120 and 1 respectively. This serum, however, was found to have a relatively high concentration of Dane particles per millilitre, and was, therefore, not truly representative of all HB_sAg positive sera. In addition, centrifugation could result in an enhancement or loss of some forms, again distorting these relative figures. Almeida (1972) estimated that the sera of most chronic carriers contained an average of 10⁵ Dane particles per millilitre with concentrations occasionally as high as 10⁸ and 10⁹. These numbers, however, are approximate as no internal standard such as latex beads was used during particle counting. It now seems certain that no correlation exists between HB_sAg titre and the number of particles per millilitre of serum (Robinson and Lutwick, 1976).

The surface of the three HB_sAg containing particles were also found to be antigenically quite complex (Levene and Blumberg, 1969; LeBouvier, 1971; Kim and Tilles, 1971; Bancroft et al, 1972). A group-specific "a" determinant was found to be common to all HB_sAg containing particles. In addition to this group-specific determinant, particles.

usually carried two type-specific determinants, a combination of either "d" or "y" and "w" or "r". Rarely, "d" and "y" determinants occurred in the same serum, either due to infection with two different antigenic subtypes (Van Kooten Kok-Doorschodt et al, 1972; Soulier and Courocicé-Pauty, 1973) or together on the same particle (Nordenfeldt and LeBouvier, 1973; Mazzur et al, 1975). Combinations of the group-specific and type-specific determinants yielded four major subtypes--adw, ayw, adr and ayr--with unequal geographic distribution (Iwarson et al, 1973; Feinman et al, 1973; Perry and Chaudhary, 1973; Mazzur et al, 1973; Dodd et al, 1973; Mazzur et al, 1974; Zuckerman et al, 1974; LeBouvier and Williams, 1975). LeBouvier (1973) observed that the ay subtype occurred predominantly in patients with acute hepatitis B, whereas ad was more prevalent among healthy carriers. Other investigators have described additional surface antigen determinants such as "b" and "c" (Levene and Blumberg, 1969) and "g" (Shorey, 1976), and it is quite probable that additional determinants may exist.

Highly purified preparations of HB_sAg have been shown to contain protein, lipid and polysaccharide. In addition, some investigators have found RNA in their preparations (Kim, 1971; Jozwiak and Koscielak, 1973) but other investigators have failed to confirm this finding (Gerin et al, 1971; Chairez et al, 1973). Six to nine polypeptides have been isolated by electrophoresis (Gerin,

1972; Chairez et al, 1973) and seven on polyacrylamide gel (Shih and Gerin, 1977). Some purified preparations of the small 22nm spherical particles have consistently been found to contain small amounts of serum protein components (Millman et al, 1971; Neurath et al, 1974), even after extensive purification. It is, as yet, not clear whether these components are normal constituents of HB_sAg or are just avidly bound to the surface antigen.

The buoyant density of the 22nm particles was found to be approximately 1.20 g/cm³ in CsCl (Gerin et al, 1969; Kim and Tilles, 1973; Gerin et al, 1975), whereas that of the Dane particle was 1.25 g/cm³ owing to its dense inner core (Kaplan et al, 1973). Following detergent treatment with NP40, the isolated empty and full cores had buoyant densities of 1.30 g/cm³ and 1.36 g/cm³ in CsCl respectively (Kaplan et al, 1976).

In 1971, Almeida et al demonstrated a second antigen-antibody system for hepatitis B distinct from HB_sAg. Following detergent treatment of Dane particles with Tween 80, a 28nm inner core was released which could be aggregated by human convalescent hepatitis B serum that was anti-HB_s negative. This new antigen was called the hepatitis B core antigen, or HB_cAg, and the antibody directed against it as anti-HB_c. Further evidence that HB_sAg and HB_cAg were antigenically distinct and that their determinants resided on separate particles was provided by Kaplan et al (1973) using radiolabelled anti-HB_c from the

first serum described by Almeida et al (1971). A Dane particle-rich preparation was sedimented in a sucrose gradient and the anti-HB_c was found to be associated with the 42nm particles by electron microscopy, and not with the HB_sAg positive fractions. Following detergent treatment with NP40, anti-HB_c activity was found to reside in the 28nm dense inner cores, the HB_cAg described by Almeida et al. Purcell et al (1974) further confirmed these findings by showing that HB_cAg detected by solid phase radio-immunoassay was present in the Dane particle preparations and not in the highly purified preparations of HB_sAg.

The hepatitis B core antigen has been seen in the nuclei of infected human (Almeida et al, 1970; Huang, 1971; Caramia et al, 1972) and chimpanzee livers (Barker et al, 1973). In 1973, Hoofnagle et al were able to purify these particles from an infected liver of an immunosuppressed chimpanzee to produce anti-HB_c in guinea pigs and to aggregate the 28nm particles with anti-HB_c containing serum.

In 1971, Hirschman et al described DNA polymerase activity in three crude pellet preparations obtained after high speed centrifugation of HB_sAg positive serum. This activity was decreased by the addition of RNAase and they concluded that this activity was probably dependent on an RNA primer template. The reaction, however, could be stimulated by the addition of poly dAT and not by poly rA-oligodT, as is typical for other RNA dependent DNA

polymerases (Temin and Baltimore, 1972). Loeb et al (1973) were also able to demonstrate DNA polymerase activity in HB_sAg positive plasma, but unlike Hirschman et al, could not decrease the activity with RNAase treatment. Kaplan et al (1973), suspecting that the DNA polymerase activity might reside in the Dane particle, prepared eight Dane particle rich pellets from eight HB_sAg-positive sera and found each to contain high levels of DNA polymerase activity.

Robinson and Greenman (1974) then demonstrated that DNA polymerase was indeed a component of the Dane particle and the cores prepared from these particles by detergent treatment. Prior to detergent treatment, whole Dane particles could only be aggregated by anti-HB_s, and not by anti-HB_c; DNA polymerase activity was present in these aggregates. After detergent treatment, the enzyme activity was found to reside in only the anti-HB_c precipitated fractions. In addition, the density of these DNA polymerase cores was found to be greater than that of DNA polymerase negative HB_cAg preparations, suggesting that the enzyme was associated with a sub-population of cores (Kaplan et al, 1973; Moritsugu et al, 1975).

The observation that DNA polymerase activity could occur in core preparations without detectable DNA or RNA led to the assumption that HB_cAg contained nucleic acid that acted as its own primer template. HB_cAg was released from Dane particles by detergent, treated with DNAase 1 to eliminate

free DNA and then disrupted with sodium dodecyl sulfate (SDS). These preparations were then examined in an electron microscope and circular double stranded DNA molecules of uniform length were seen (Robinson et al, 1974). When the DNAase pretreatment step was eliminated, linear DNA molecules with varying lengths were seen in addition to the circular forms. This suggested that the circular DNA molecules were in a protected position within the core whereas the linear DNA molecules were not. The origin of these linear molecules is unclear, but it is conceivable they are soluble DNA forms present in the plasma of all patients (Kamm and Smith, 1972).

The mechanism of action of the DNA polymerase is not clear but it appears that replication of the circular DNA molecule probably does not occur in the Dane particle because of limited space (Robinson and Lutwick, 1976). In addition, natural RNA and DNA as well as polyribonucleotides and polydeoxyribonucleotides do not seem necessary to stimulate the reaction, nor do high concentrations of RNAase inhibit it, as suggested by Hirschman et al (1971). It seems apparent that the DNA polymerase described by Kaplan et al (1973) and Robinson and Greenman (1974) differs markedly from that described by Hirschman et al (1971), and raises the possibility that the DNA polymerase described by the latter may be of host origin.

A third antigen-antibody system was described in 1972 by Magnus and Espmark who observed a precipitating line

between two HB_sAg-positive sera. This new antigen was found to be distinct from the previously described HB_sAg and HB_cAg systems and was designated the "e" determinant or HB_eAg. Unlike the previously described "a", "d", "y", "w" and "r" determinants which resided on the HB_sAg particle, the "e" determinant appeared to be associated with some other component, or particle, of HB_sAg positive serum. Equilibrium centrifugation showed that this new antigen had a buoyant density of 1.29 g/cm³ in CsCl and a 12S sedimentation coefficient (Magnius et al, 1975; Magnius, 1975). These values indicated that HB_eAg was considerably smaller than the HB_sAg particle.

The "e" antigen has been found exclusively in HB_sAg positive sera while its antibody, anti-HB_e is present in both HB_sAg-positive and anti-HB_s positive sera (Magnius et al, 1975). Studies by several investigators have led them to suggest that the presence of HB_eAg in serum may have prognostic implications for patients with hepatitis B (Nielsen et al, 1974; Magnius et al, 1975; Eleftheriou et al, 1975; Feinman et al, 1975; El Sheikh et al, 1975; Maynard et al, 1976; Nordenfelt et al, 1975; Smith et al, 1976; McAuliffe et al, 1976; Trepo et al, 1976; Takahashi et al, 1976). The detection of HB_eAg in HB_sAg-positive diseases such as chronic active hepatitis and cirrhosis is quite frequent and appears to indicate a poor prognosis. Conversely, the presence of anti-HB_e, which is found most often in healthy HB_sAg carriers appears to indicate a more

favourable outcome (Okada et al, 1976). Asymptomatic HB_sAg carriers were generally found to have a low frequency of HB_eAg (Sherlock, 1976).

The early reports of Magnius and Espmark (1972a, b) and subsequent reports by other investigators (Nordenfelt and Kjellen, 1975; Schweitzer et al, 1975; Takahashi et al, 1976; Okada et al, 1976; Magnius et al, 1975; Skinhøj et al, 1976) have suggested that the presence of HB_eAg in the serum of HB_sAg carriers correlated with infectivity. A number of studies have linked the detection of HB_eAg in serum with the presence of Dane particles (thought to be the hepatitis B virus) in serum (Takahashi et al, 1976; Nordenfelt and Kjellen, 1975; El Sheikh et al, 1975; Hindman et al, 1976; Okada et al, 1976; Nordenfelt and Andrén-Sandberg, 1976; Werner et al, 1977; Tong et al, 1977; Zanetti and Ferroni, 1977), with elevated levels of DNA polymerase (thought to be a marker for viral replication) in serum (Nordenfelt and Kjellen, 1975; Hindman et al, 1976; Nordenfelt and Andrén-Sandberg, 1976; Maynard et al, 1976; Alter et al, 1976; Imai et al, 1976; Zanetti and Ferroni, 1977; Tong et al, 1977) and with HB_cAg in serum (Takahashi et al, 1976) and in liver (Takahashi et al, 1976; Murphy et al, 1976; Trepo et al, 1976; Trepo et al, 1978).

The relationship of HB_eAg to infectivity has been supported by transmission data. Okada et al (1976) showed that all 10 infants born to HB_sAg and HB_eAg positive

mothers developed HB_sAg whereas none of 7 infants born to HB_sAg and anti-HB_e mothers did. Beasley et al (1977) also reported that maternal HB_e antigenemia was a good predictor of vertical transmission. In addition, studies of accidentally inoculated medical personnel have also shown an apparent correlation between HB_eAg and infectivity (Grady, 1976; Alter et al, 1976).

There is, however, some question about the lack of infectivity of HB_sAg and anti-HB_e positive sera. Magnus et al (1975) reported that 10 of 12 healthy blood donor carriers of HB_sAg and anti-HB_e donated a total of 95 units of blood with no reported cases of post transfusion hepatitis. Other investigators have reported that there are no Dane particles in anti-HB_e positive serum (Imai et al, 1976; Hindman et al, 1976). Many investigators, however, have found this not to be the case. El Sheikh et al (1975) and others (Nordenfelt and Kjellen, 1975; Zanetti and Ferroni, 1977; Werner et al, 1977) found Dane particles in some HB_sAg, anti-HB_e positive sera. Similarly, infection could also be transmitted with anti-HB_e positive sera. Berquist et al (1976) inoculated 4 chimpanzees with HB_sAg anti-HB_e positive sera containing no detectable Dane particles. They found a human serum with anti-HB_e was infectious for one chimpanzee and that a chimpanzee serum with anti-HB_e may have caused infection in a second animal. In addition, there have been reports of infants born to HB_sAg anti-HB_e positive mothers developing HB_s antigenemia,

albeit transiently in 2 of the 3 studied (Schweitzer et al, 1975; Gerety, 1976).

Even though HB_eAg has been characterized biophysically (Magnius, 1975), its origins and relationship to HB_sAg and HB_cAg remain unknown. Its close association with DNA polymerase prompted one group of investigators to suggest that it was the DNA polymerase itself (Melnick et al, 1976). This speculation was quickly refuted by Neurath and Strick (1976). Vogten et al (1976) suggested it was the result of an impaired host immune response to hepatitis B infection. Neurath and Strick (1977) concurred and proposed that the "e" antigen was, in reality, an abnormal immunoglobulin G. This, too, has been refuted (Takahashi et al, 1978). Another suggestion is that HB_eAg is a soluble host protein released in response to hepatitis B infection. A fourth possibility is that HB_eAg is a matrix protein, produced during viral replication (hence its association with DNA polymerase and Dane particles) to bind HB_sAg at the hepatocytic nuclear membrane to the 28nm cores packaged in the nucleus (Gerin, personal communication).

Researchers also differ as to where the "e" antigen is located, relative to the Dane particle. Neurath et al (1976) identified HB_eAg on the surface of this particle while Lam et al (1977) claim to have released HB_eAg from within the Dane particle by detergent treatment. Subsequently, it was shown that the Dane particle preparation from which Lam et al released HB_eAg was contaminated with anti-HB_c and this has raised some serious doubts about the

significance of their findings (Huang, personal communication).

Whatever its origins, the "e" antigen is apparently not one antigen but a group of antigens. In 1976, Williams and LeBouvier noted two precipitation lines between two HB_sAg-positive sera and designated them "e₁" and "e₂". These two lines could appear either simultaneously or sequentially during the course of an acute hepatitis B illness, and prompted these investigators to speculate that the appearance of "e₂" after "e₁" was a sign of impending resolution of the disease. This theory remains to be confirmed.

In addition to acute hepatitis and chronic carriage of the surface antigen, HB_sAg has been implicated in chronic active hepatitis (Gitnick et al, 1969; Blumberg et al, 1970; Krassnitsky et al, 1970; Reinicke and Nordenfelt, 1970; Velasco and Katz, 1970; Prince et al, 1970), cirrhosis (Maynard et al, 1970; Gocke and Kavey, 1969; Wright et al, 1969; Fox et al, 1969; Kaplan and Grady, 1971), periarteritis nodosa (Gocke et al, 1970, Prince and Trepo, 1974) and carcinoma of the liver (Smith and Blumberg, 1969; Vogel et al, 1970; Hadziyannis et al, 1970; Prince et al, 1970; Moertel et al, 1970; Dennison et al, 1971; Anthony, 1973; Shikata, 1973; Reed et al, 1973; Maupas et al, 1975; Hadziyannis, 1975; Tabor et al, 1977). A course of events for acute hepatitis B is presented in figure 1 (Redeker, 1975).

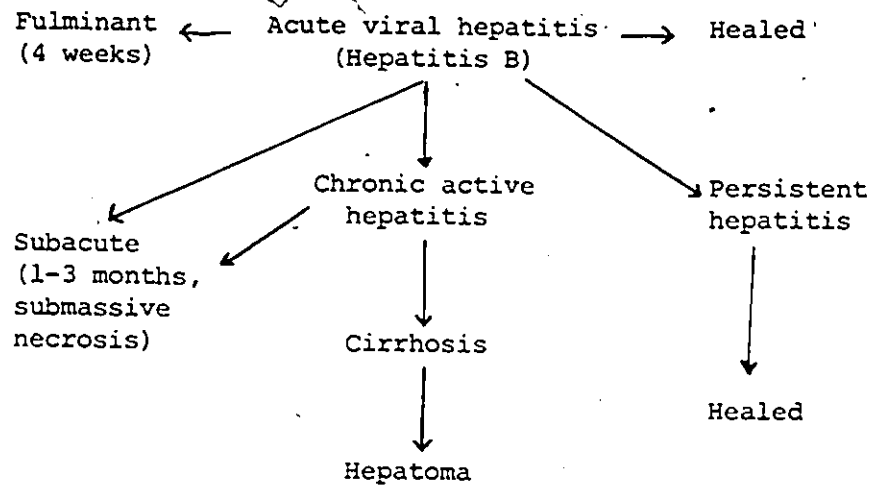


Figure 1:-- Courses of Acute Viral Hepatitis

Immune complexes have been detected in chronic active hepatitis and polyarteritis nodosa, but the pathogenic role of these HB_sAg-containing complexes is still not understood (Gerber et al, 1972; Prince, 1971; Wright et al, 1969).

A strong correlation exists between the occurrence of joint and skin manifestations in patients with acute hepatitis B and decreased levels of complement (C3), suggesting that clinical symptoms may be caused by circulating immune complexes (Onion et al, 1971). Similar decreases in C3 were also reported by Grob et al (1971) and Alpert et al (1971). In a more recent study, Duffy et al (1976) showed that this disease can cause not only arthralgias but true polyarthrititis which can simulate rheumatoid arthritis.

The relationships of HB_sAg, HB_cAg, HB_eAg and their respective antibodies and DNA polymerase to the course of an acute hepatitis B infection are shown in figure 2 (adapted from Krugman et al, 1974).

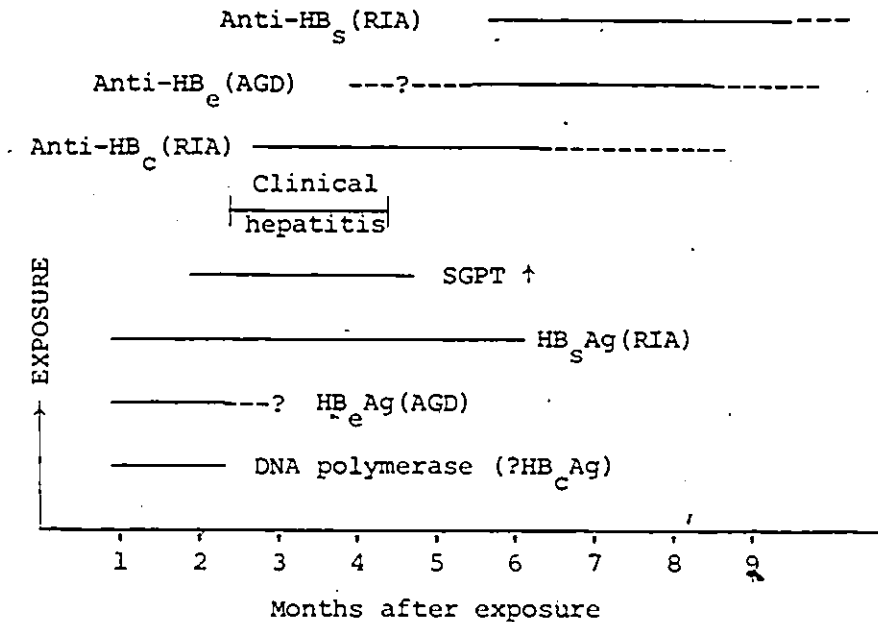


Figure 2: Course of an acute HB_sAg infection.

DNA polymerase activity and HB_eAg have been detected in the serum of patients with HBV infection after the appearance of HB_sAg but before or at the time of biochemical evidence of liver disease (Smith et al, 1976; Norkrans et al, 1976). This enzyme was found to be transient in patients with acute hepatitis B but could persist for months and even years in patients with chronic liver disease. HB_sAg is detectable throughout the clinical

course of the illness, often disappearing within 6 months of exposure and within 3 to 4 months after the onset of jaundice. In healthy carriers and chronic disease, this antigen can persist for many years. Anti-HB_c appears one or more months after the onset of HB_s antigenemia and is closely associated with the onset of clinical symptoms (Krugman et al, 1974). The anti-HB_c titre may persist or gradually decrease with recovery and disappearance of HB_s Ag. In patients with chronic liver disease, high anti-HB_c titres are common and can persist for very long periods of time (Hoofnagle et al, 1973; Hoofnagle et al, 1974). Anti-HB_e is usually not detected during clinical illness, perhaps due to the insensitivity of the detection system used, and is occasionally detected during convalescence (Smith et al, 1976). In contrast to the early appearance of anti-HB_c, anti-HB_s appears several weeks or even months after the disappearance of HB_s Ag. This sequence of events has been confirmed not only in chimpanzees (Hoofnagle et al, 1973), but also in humans (Purcell et al, 1973).

The prevention of hepatitis B infection and disease, and transmission of HB_s Ag has been the major aim of all clinicians. Using disposable needles and syringes, whenever possible and sterilizing all medical equipment has greatly reduced its iatrogenic incidence. In addition, it has also been a policy not to accept blood from persons who have had the disease or from high risk populations.

such as prisoners, residents of mental institutions and homosexuals (Melnick et al, 1976). A third method has involved the use of frozen red blood cells for transfusions (Tullis et al, 1970). Removal of the plasma and washing the red cells prior to use have appeared to sufficiently reduce the concentration of HB_sAg and to prevent clinical disease (Werch et al, 1971; Huggins et al, 1973).

Passive and active immunization procedures have gained much attention recently as potentially useful approaches to disease attenuation and prevention. Studies on passive immunization using a specific hepatitis B hyperimmune gamma globulin (HBIG) have been encouraging. HBIG was prepared from plasma containing anti-HB_s titres 50,000 times greater than found in commercially available gamma globulin (Prince et al, 1971; Szmunness et al, 1974). When this HBIG was administered to 10 children 4 hours after exposure to HB_sAg, 6 developed no disease and 1 had transient antigenemia and abnormal SGOT levels for 6 days--a 70% level of protection. All 11 control children who received the same dose of virus but without HBIG developed characteristic hepatitis B infection (Krugman et al, 1971).

Early experimentation with active immunization consisted of a series of human studies using heated inactivated HB_sAg-positive sera (Krugman et al, 1970; Soulier et al, 1972). Next, successful immunization of chimpanzees with a purified, formalin-inactivated HB_sAg vaccine was reported;

in addition, these animals were able to withstand a virus challenge (Hilleman et al, 1975; Purcell and Gerin, 1975). More recently still, a 2 year study of the efficacy of a hepatitis B vaccine showed it to be highly effective in protecting patients and staff in high risk hemodialysis units (Maupas et al, 1978). This group also found that if the inactivated HB_sAg vaccine contained aluminum hydroxide as adjuvant; the result was a more rapid and stronger anti-HB_s response.

Many arguments have been advanced against the use of HB_sAg of human origin as a vaccine source (Zuckerman, 1976). The principal objection is based on the presence in the majority of HB_sAg preparations of protein contaminants originating from serum (Gerin et al, 1971; Millman et al, 1971). This contamination could be the result of either insufficient purification of the antigen (Goudeau et al, 1974), or from integration of certain host proteins in the structure of the virus itself (Neurath et al, 1974); this latter assertion, however, has not yet been clarified (Schuurs and Wolters, 1975).

This unconventional method for preparing a hepatitis B vaccine could originally be justified by the inability to successfully grow the hepatitis B virus in tissue culture systems (Zuckerman and Earl-Baines, 1973; Maupas et al, 1976). The recent discovery of HB_sAg and Dane particle replication in a hepatoma cell line (Alexander et al, 1978) may mean that a potential source of contaminant-free HB_sAg has now become available for vaccine preparation. It remains

to be seen, however, if this is indeed a suitable source of hepatitis B antigen.

Another possible solution to the potential problem of human serum contaminants in conventionally prepared HB_sAg-vaccines has been the finding that disrupted HB_sAg particles yield low molecular weight polypeptides, retaining specific HB_sAg determinants on their surface (Rao and Vyas, 1973; Howard and Zuckerman, 1975). This finding has been the basis for preparing a hepatitis B subunit vaccine. Melnick et al (1976) have prepared two vaccines using glycoproteins from purified HB_sAg particles with molecular weights of 22,000 and 25,000 respectively; moreover, since these glycoproteins are free of nucleic acids, these vaccines have been considered to be incapable of infecting the recipient. Subunit vaccines have been found to be highly immunogenic in chimpanzees, and they may provide a safe way to actively protect recipients without raising the spectre of an autoimmune response induced by human serum contaminants in conventionally prepared vaccines (Zuckerman, 1976).

II. Cell Mediated Immunity

Two distinct lymphocyte populations are known to exist in normal, healthy humans. These have been designated thymus-derived or T lymphocytes and bone-marrow-derived or B lymphocytes. T and B lymphocytes can be distinguished by a variety of criteria, including surface characteristics,

ontogeny, life span, location in lymphoid tissue and immunologic function. When T or B cells are exposed to antigens or other stimuli, a complex series of alterations in cellular transport and metabolism occur. This process, known as lymphocyte activation, begins within minutes of exposure to the stimulus, continues for several days and leads to changes in cell morphology, to increases in DNA, protein and RNA synthesis and often to mitosis and cell division (Oppenheim and Rosenstreich, 1976; Wedner and Parker, 1976). As the response progresses, the cells synthesize new enzymes and begin to secrete a variety of biologically active macromolecules called lymphokines. The B cells eventually differentiate into antibody producing plasma cells while the T lymphocytes mediate the cellular immune response (Craddock et al, 1971).

T and B lymphocytes differ in their requirements for activation by antigens. Protein and carbohydrate antigens apparently activate B cells indirectly by combining with specific immunoglobulin receptors on the B cell surface, whereas polymerized antigens can activate B cells directly. Most antigens, however, are ineffective B cell activators unless a second signal, thought to be a protein or group of proteins from activated T cells, is also present (Wedner and Parker, 1976).

T lymphocytes also respond to a variety of antigenic and mitogenic stimuli, usually faster than similarly activated B cells. T cells are thought to contain

recognition units for antigen, but their molecular character is poorly understood. Like B-cell activation, T lymphocyte stimulation also requires a soluble helper protein, known as lymphocyte activation factor, which is released by macrophages (Oppenheim and Rosenstreich, 1976). Additional factors, including serum proteins, transfer factor, interferon and other lymphokines are probably also involved in this response (Parker, 1976).

The T lymphocyte population of cells is a heterogeneous one consisting of functionally discrete subpopulations of T cells (Cantor and Boyse, 1975; Huber et al, 1976). These subpopulations probably respond to antigenic stimuli in different ways, either by directly stimulating or inhibiting the response, or maturation and modulation of lymphocyte function.

The cell-mediated immune response, which is a function of the T lymphocyte, is the major host response to a variety of obligate intracellular parasites, allografts, tumors and delayed type hypersensitivity (David, 1966; Mackaness and Blanden, 1967; Hall, 1967; Hellstrom and Hellstrom, 1969; World Health Organization, 1973). The direct measurement of the effector mechanism directed against these agents has often been difficult in man. Estimations of the cell-mediated immune (CMI) response, however, are possible by measuring various other CMI expressions. These include the in vivo detection of delayed-type hypersensitivity (DTH) and in vitro tests such as

antigen-specific lymphocyte transformation (LT), lymphocyte-mediated cytotoxicity and assays of lymphokine secretion such as migration inhibition factor, transfer factor and interferon production. Protective immunity, DTH, LT and MIF production are all functions of the T-cell and a host often exhibits several of these manifestations simultaneously (Lefford, 1975). This has led some investigators to conclude that a positive DTH, LT or MIF test denotes protective immunity. Unfortunately, this is not the case as dissociations of these CMI parameters can occur either spontaneously or experimentally, indicating that they are unreliable markers for protective immunity (Youmans, 1975).

It has been proposed that the host response to hepatitis B infection is largely a function of the T lymphocyte (Dudley et al, 1972a, b; Giustino et al, 1972; Popper et al, 1972). Dudley et al (1972a) postulated that if the infected individual has an adequate CMI response to the hepatitis B virus, he will develop clinical liver disease and eventually recover. If, on the other hand, the response is deficient, there will be little or no liver disease and the individual will become a chronic carrier of HB_sAg. The persistence of HB_sAg in serum is frequently associated with the development of chronic active and chronic persistent hepatitis (Nielsen et al, 1971), and may eventually lead to hepatic carcinoma (Redeker, 1975).

The CMI response to hepatitis B infection has been studied by various in vivo and in vitro techniques. These

include:

- (1) Delayed hypersensitivity testing in humans and in animal models;
- (2) PHA stimulation studies of lymphocytes from patients with hepatitis B positive liver disease;
- (3) Lymphocyte stimulation and transformation studies;
- (4) Migration inhibition studies; and
- (5) Lymphocyte cytotoxicity studies.

Delayed hypersensitivity testing (DHT) in man has been somewhat limited because of the very real danger of transmitting hepatitis B to test recipients by intradermal injections of hepatitis B antigens. Some investigators have claimed that this in vivo CMI response can be correlated to an in vitro test, the production of MIF (Irwin et al, 1974). Other investigators, however, have found this correlation to be faulty, demonstrating that one CMI response (DHT) may occur independently of another (Trepo et al, 1975). In 1950, Henle et al reported skin reactions in humans to amniotic fluid from eggs inoculated with the presumed infectious hepatitis virus. Later, other investigators attempted to demonstrate DHT in 10 patients with various liver diseases by intradermally

inoculating them with autologous liver suspensions. Even though all liver biopsies had histological evidence of an inflammatory response, no correlation could be made between these responses and the resulting cutaneous reactions (Geduldig and Iber, 1962). Other researchers demonstrated that the positive skin test to dinitrochlorobenzene (DNCB) remained intact, despite HB_sAg-positive chronic active or acute hepatitis (Bolin et al, 1973; Kanakoudi-Tsakalidis, 1974). More consistent and interesting data, however, have been obtained from animal experimentation. Previously sensitized guinea pigs (Irwin et al, 1972; Gerety et al, 1974) and chimpanzees (Ibrahim et al, 1974; Trepo et al, 1975), with anti-HB_s and anti-HB_c titres, had positive skin tests when challenged with various purified hepatitis B antigens. The correlation between DHT and hepatitis B antibodies, a humoral immune response was consistently greater than between DHT and other in vitro CMI parameters.

Viral inhibition of the in vitro lymphocyte response to PHA was described by Montgomery et al in 1967. Similar results were obtained when lymphocytes from infectious hepatitis (Mella and Lang, 1967; Martini et al, 1970) and hepatitis B patients were stimulated with PHA (Willems et al, 1969). The findings for hepatitis B were subsequently confirmed by many other investigators (Agarwal et al, 1971; Millman et al, 1971; Baroyan et al, 1970; Brooks, 1972; Malacarne and Dallapiccola, 1973;

Jöbsis, 1973; Fox et al, 1973; Campion and Wangel, 1975). Homologous sera obtained from patients with acute viral hepatitis, alcoholic hepatitis, primary biliary cirrhosis, asymptomatic HB_s antigenemia and halothane hepatitis were also found to inhibit PHA proliferation of lymphocytes obtained from healthy individuals (Mella and Taswell, 1970; Hsu and Leevy, 1971; Brooks, 1972; Newberry et al, 1973; Fox et al, 1973; Macsween and Thomas, 1973; Clot et al, 1973; Pétri and Mészáros, 1973). Lacava (1970) also noted such a decrease with sera from acutely ill hepatitis B patients but when the lymphocytes were incubated in sera from healthy HB_sAg-positive carriers, no such suppression occurred. Sutnick et al (1973), on the other hand, found no significant difference in the levels of tritiated thymidine (³H-T) incorporation by PHA-stimulated lymphocytes of HB_sAg-positive people and normal controls and concluded that HB_sAg did not impair the lymphocyte response to PHA.

Results of lymphocyte stimulation (or transformation) and migration inhibition tests have, by and large, been equivocal. Liver extracts were prepared to study lymphocyte transformation and results were considered positive if a certain percentage of the cells underwent blast transformation. Lymphocytes from patients with chronic active hepatitis were converted to blasts more often (Tobias et al, 1967; Bacon et al, 1971; Eddelston, 1974)

by these hepatic antigens than were those from patients with acute disease (Tobias et al, 1967; Smith et al, 1972). Judmaier et al (1972) measured the incorporation of $^3\text{H-T}$ into lymphocytes from patients with liver disease and noted a positive correlation between the rate of $^3\text{H-T}$ uptake and the degree of liver damage (a measure of serum transaminases--SGOT and SGPT).

Lymphocyte stimulation assays using crude and purified HB_sAg preparations have also yielded equivocal results. Reports of in vitro lymphocyte stimulation using cells from convalescent patients (Yeung Laiwah, 1971; Pettigrew and Russell, 1972; Sodomann and Havemann, 1973; De Gast et al, 1973; Yeung Laiwah et al, 1973; Reisenbuk and Ioks, 1974; Koszinowski et al, 1974; Chandra, 1975; Tong et al, 1975) are opposed by findings of non-stimulation and even inhibition (Wands et al, 1975; Tiku et al, 1978). Similarly, reports of stimulation with HB_sAg in chronic active (Tong et al, 1975; Tiku et al, 1978) and acute hepatitis (Tiku et al, 1978) are contrasted by findings of non-stimulation in these groups (Yeung Laiwah et al, 1973; Pettigrew and Russell, 1972; Koszinowski et al, 1974). Combinations of HB_sAg and PHA have been reported to be both stimulatory (Tsuji et al, 1972) and non-stimulatory (Barinsky et al, 1975).

Purified and crude liver preparations have produced both inhibition (Verganni et al, 1972; Miller et al, 1972; Knolle et al, 1973) and non-inhibition (Zauli et al,

1974) of leukocyte migration in chronic active hepatitis. Similarly, using crude and purified HB_sAg preparations, both inhibition (Gerber et al, 1973; Knolle et al, 1973; Gerber et al, 1974; DeMoura et al, 1975; Irwin et al, 1974) and stimulation (Frei et al, 1973; Erard, 1974; Erard et al, 1974) of migration of leukocytes have been reported for acute hepatitis B infection; with recovery, either no reactivity (Gerber et al, 1973; Gerber et al, 1974; Ibrahim et al, 1974) or inhibition of migration (Yeung Laiwah et al, 1973; Frei et al, 1973; Erard et al, 1974) have been described. Inconsistencies in assessing migration inhibition in chronic active hepatitis patients also persist; it has varied from 0% (DeMoura et al, 1975) to 50% (Dudley et al, 1972).

The fifth and most consistent in vitro technique for assessing the CMI response to hepatitis B has been the lymphocyte cytotoxicity assay. Sensitized lymphocytes destroy Cr⁵¹ labelled cells and the amount of radioactive release is a measure of T-cell function (Wands et al, 1975; Wands and Isselbacher, 1975; El Sheikh et al, 1978). This procedure, however, is technically the most difficult.

III. Objectives

The major objectives of the present study were:

- (1) To study the epidemiology of HB_eAg and anti-HB_e in various HB_sAg-positive populations.

- (2) To measure DNA polymerase activity and to assess specificity of this enzyme in HB_SAg-positive sera.
- (3) To establish a relationship between DNA polymerase, the "e" antigen/antibody system and the putative hepatitis B virion, the Dane particle.
- (4) To measure the in vitro cell-mediated immune responses (lymphocyte stimulation and migration inhibition) of persons who have recovered from hepatitis B infection (post hepatitis B) in order to resolve the equivocal data that presently exist.

The enabling objectives of this study were:

- (1) To evaluate techniques for detecting HB_SAg and anti-HB_S in sera.
- (2) To study the epidemiology of HB_SAg and anti-HB_S in various populations.
- (3) To devise a simplified and inexpensive technique for the purification of HB_SAg.
- (4) To assess the homogeneity and purity of the final purified HB_SAg preparations.
- (5) To select appropriate sera and tissues for

HB_cAg isolation and purification.

- (6) To determine optimal in vitro conditions for lymphocyte cultures and to assess the effect of variables (age, pH, etc.) on the in vitro lymphocyte stimulation response.

MATERIALS AND METHODS

I. Antigen Studies

A. Sources of plasma and sera

Plasma and sera for hepatitis B surface antigen (HB_sAg), core antigen (HB_cAg), e antigen (HB_eAg) and their respective antibodies were obtained from: (1) Drs. F. S. Mazzur, N. Nath and R. Y. Dodd, American Red Cross, Bethesda, Md.; (2) Drs. J. L. Gerin and P. Kaplan, MAN Program, Oak Ridge National Laboratory, Rockville, Md.; (3) Dr. J. H. Hoofnagle, Bureau of Biologics, Division of Blood and Blood Products, Bethesda, Md.; (4) Drs. R. H. Purcell and V. J. McAuliffe, NIAID, Bethesda, Md.; (5) Dr. M. Goldfield, State of New Jersey Dept. of Health, Trenton, N.J.; (6) Dr. L. Overby, Abbott Laboratories, N. Chicago, Ill.; (7) Rideau Regional Centre, Smith Falls, Ontario; (8) Huronia Regional Centre, Orillia, Ontario; (9) Ottawa General Hospital, Ottawa, Ontario; and (10) Ottawa Civic Hospital, Ottawa, Ontario.

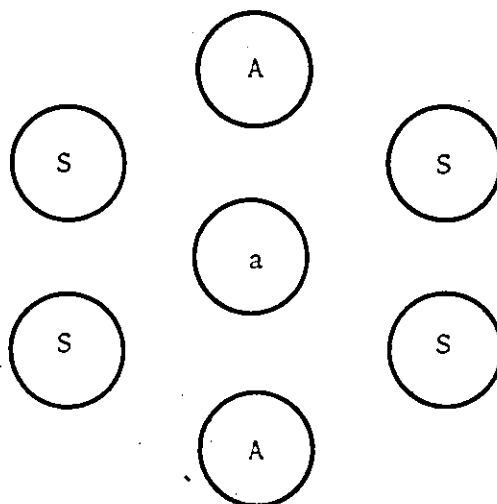
B. Hepatitis B surface antigen and antibody

1. Detection of HB_sAg

Plasma and sera were tested for HB_sAg and anti- HB_s by one or more of three methods. These were agarose gel

diffusion (AGD), counterimmunoelectrophoresis (CIEP) and radioimmunoassay (RIA).

a) AGD:--Glass slides (2.5 cm x 7.5 cm) were precoated with 0.2% agarose (Indubiose A45--Fisher Scientific, Fairlawn, N.J.) and allowed to dry. Two and one half millilitres of 0.8% A45 agarose in an immunodiffusion buffer (0.05M Tris-0.15M saline-0.1% sodium azide, pH 7.6) were poured onto each slide and allowed to harden at 4°C. A six-shooter pattern (figure 3) was cut in the



hardened gel (well \emptyset 3.0mm) and the agarose plugs removed by gentle suction. The gaps between the well and the slide were sealed with a drop of hot 0.8% agarose A45 and the excess liquid agarose was aspirated out by gentle suction.

The "A" wells were filled with a known HB_sAg-positive reagent while the four remaining "S" wells were filled with unknown plasma and/or sera samples. The centre "a" well was filled with a known anti-HB_s-positive

reagent. All wells were refilled with their initial reagents 1 hour later and then placed in a moist chamber. These chambers were incubated at room temperature for 48-72 hours, at which time the slides were read. Slides were kept moist for ~~up to~~ 1 week for periodic rereading.

b) CIEP:--Microscope slides (2.5 cm x 7.5 cm) were precoated with 0.2% A45 agarose. A tank and gel buffer were then made up as follows from these stock solutions:

Solution A--Barbital 2.763g/L. deionized water

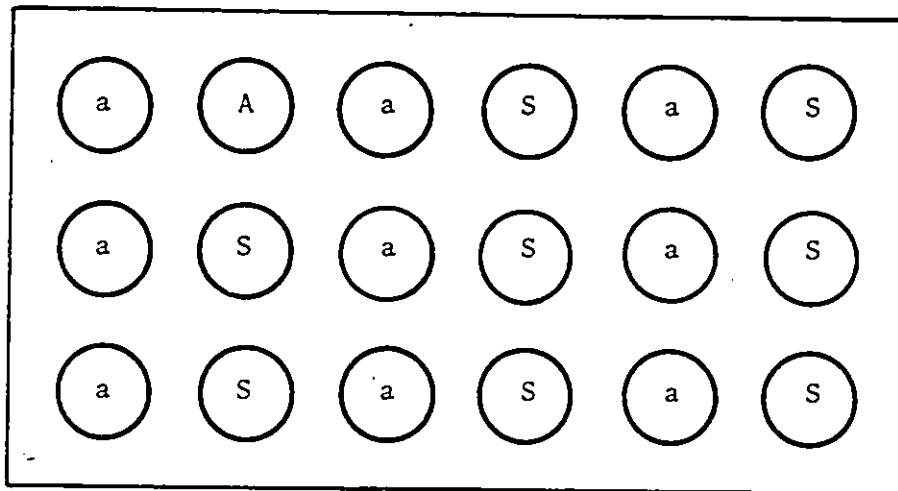
Solution B--Sodium Barbitol 103.0g/L. deionized
water

Solution C--NaCl 29.23g/L. deionized water

109 ml of solution A, 12.5 ml of solution B and 187.5 ml of solution C were combined with deionized water to approximately 2 litres. The pH was adjusted to 8.6 with 1N NaOH. Fifty millilitres were used for preparation of the gel.

Agarose A45 was dissolved in the 50 ml of buffer to a concentration of 0.8%. Each slide received 2.5 ml and was allowed to harden at 4°C. Parallel rows of wells (well Ø 4.5mm) were punched on each slide to a maximum of 18 wells. The agarose plugs were removed by gentle suction and the gel-glass gap sealed with a drop of hot agarose as previously described. The "A" well was filled with a known HB_sAg-positive reagent, the "S" wells were filled

with unknowns and the "a" wells were filled with a known anti-HB_s serum (figure 4).



All wells were refilled within one hour with their respective starting materials. The slides were then placed in an electrophoretic cell (Buchler Instruments, Fort Lee, N.J.) containing the barbital-barbitol-saline buffer as described. Cotton strips were attached to the positive and negative poles of each slide and placed in the buffer. A 12 milliamp current was allowed to flow through each slide for 75 minutes, after which time the slides were read.

c) RIA:--A commercially available kit, Ausria II (Abbott Laboratories, N. Chicago, Ill.) was used for detecting HB_sAg and anti-HB_s in sera and plasma. Positive and negative controls were included in each group of unknown sera and/or plasma tested. Samples were counted in a Nuclear Chicago model 1100 gamma scintillation counter and expressed as counts per minute (cpm). Samples with values greater than or equal to 2 times the mean of the negative controls were considered positive for

HB_sAg (P/N \geq 2).

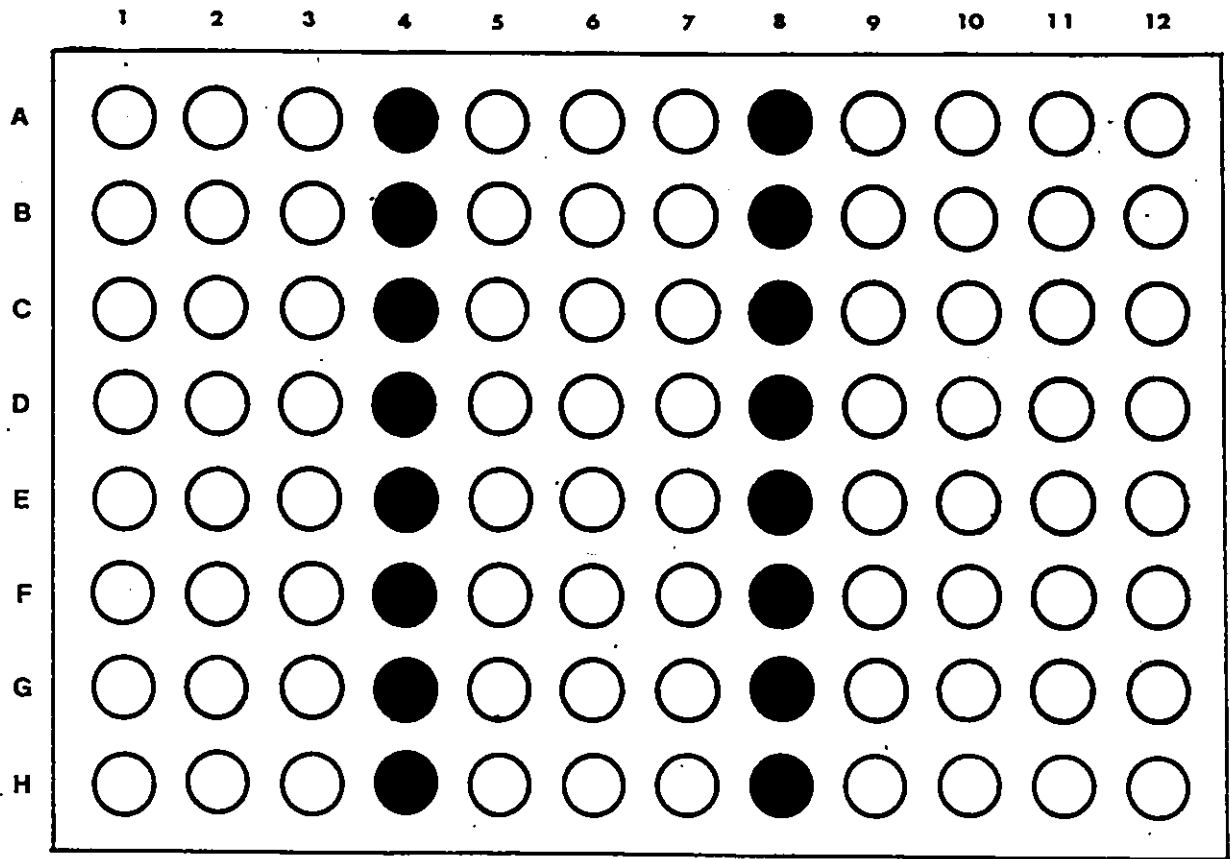
d) Sera tested:--A panel of 20 sera positive for HB_sAg by RIA and a panel of 20 negative sera were obtained from the American Red Cross for confirmatory testing by AGD, CIEP and RIA. In addition, 195 consecutive sera from Down's syndrome (DS) patients and 2,400 consecutive sera from other mentally retarded (OMR) residents of the Rideau Regional Centre were collected over a 4 year period and assayed for HB_sAg by RIA in conjunction with the Provincial Laboratory, Bell's Corners, Ontario to determine the incidence of the antigen in an institutionalized population.

2. Detection of anti-HB_s by the passive hemagglutination assay (HA)

a) Reagents:--Human group O Rh-negative red blood cells coated with subtype ad or ay antigen by the chromic chloride technique and control cells (in PBS) were purchased (Electronucleonics, Bethesda, Md.). TAP buffer (PBS, pH 7.4 containing 0.5% bovine serum albumin, 0.0025% polyvinyl pyrrolidone, 1:20,000 Tween 80 and 1:1000 sodium azide) was obtained from the same company and served as a diluent.

b) Anti-HB_s assay:--Serial 2-fold dilutions (to 1:8) of the sera to be tested were made with 25 μ l loops into 25 μ l TAP buffer in polyvinyl V-well microtitre plates (figure 5).

Figure 5



After 30 minutes incubation at room temperature, 25 μ l of the appropriately coated erythrocytes were added. Rows 1, 2 and 3 received subtype ad or ay coated cells; rows 5, 6 and 7 received non-coated control cells. Rows A9-12 and B9-12 contained TAP buffer only and either ad or ay coated cells added to serve as controls. The plates were covered with cellophane and incubated for an additional 30 minutes at room temperature. They were then centrifuged at 1200 rpm for 10 minutes, tilted at an angle of 70° for 15 minutes and read. A streaming or inverted umbrella-shaped red cell pattern was considered positive hemagglutination, and thereby anti-HB_s-positive.

c) Sera tested:--A panel of 20 sera positive for anti-HB_s by RIA and a panel of 20 sera negative for anti-HB_s were obtained from Dr. R. H. Purcell for confirmatory testing by HA. In addition, 195 consecutive sera from DS patients and 2400 consecutive sera from OMR residents of the Rideau Regional Centre collected over a four year period were assayed for anti-HB_s by HA to determine the incidence of this antibody in an institutionalized population.

3. Subtype determination

a) Populations studied:--One hundred consecutive sera positive for HB_s Ag by CIEP from each of 4 distinctive populations were subtyped by AGD. These populations were:

- (1) An institutionalized population--Rideau Regional Centre, Smith Falls, Ontario;
- (2) An acutely ill population--Ottawa Civic and Ottawa General Hospitals;
- (3) A donor population--American Red Cross, Bethesda, Md.; and
- (4) Twelve HB_s Ag positive sera from a renal dialysis unit--Ottawa General Hospital.

b) AGD technique:--A 6-shooter pattern (figure 3) was cut in 0.8% agarose A45. The "A" wells were filled with either a known ad or ay antigen positive serum

(courtesy Dr. G. LeBouvier, Yale University, New Haven, Ct.) while the four peripheral "S" wells received subtype-unknown HB_sAg positive samples. The "a" well was filled with a known anti-ad, ay, d or y antiserum (courtesy Dr. G. LeBouvier and Dr. L. Overby). All wells were refilled twice within the first few hours after the initial filling and incubated in a moist chamber at room temperature for 48-72 hours. Slides, were kept for up to 1 week for periodic re-reading.

4. Purification of HB_sAg

Plasma samples were converted to sera by adding 1M CaCl₂ (3 ml/100 ml) and incubating at 56°C for 1 hour. The sera were then filtered through cheesecloth and clarified at low speed centrifugation (3500 rpm) in an IEC centrifuge (Needham Heights, Mass.) for 30 minutes.

a) Centrifugation:--All high speed centrifugations were done in a Beckman L3-50 ultracentrifuge at 5°C.

b) Gradient solutions:--A 1.86 g/cm³ stock solution of cesium chloride (CsCl, Harshaw Chemicals, Solon, Ohio) was prepared in 0.01M Tris-HCl, pH 7.4 from which the various CsCl solutions of varying density were made. Varying sucrose solutions were prepared from a stock 66% w/w solution (Schwarz-Mann, Orangeburg, N.J.).

c) Preparation of HB_sAg starting material for purification:--The clarified serum was centrifuged at 21,000 rpm for 4 hours in a fixed angle 30 rotor (Spinco,

Palo Alto, Calif.). The supernatant was decanted into a sterile container to be used as the starting material for HB_sAg purification. Two millilitres of phosphate buffered saline (PBS, pH 7.4) were added to each tube and the pellets resuspended. The suspensions were combined and stored at -70°C for later studies.

d) Large volume purification:--This procedure was done in conjunction with Dr. J. L. Gerin and consisted basically of using large volume rate-zonal rotors for purification and ultraviolet (UV) spectrophotometry at 280nm and RIA for monitoring the antigen. This procedure, which was a modification of a technique described by Gerin et al (1971), is summarized in figure 6.

- (1) First isopycnic banding--Step 1. An empty Ti-14 rotor with a B29 liner rotating at 3000 rpm was edge loaded with 110 ml of the HB_sAg starting material, followed by 250 ml of 1.20 g/cm³ CsCl and 200 ml of 1.40 g/cm³ CsCl to fill. Fifty millilitres of 0.01M Tris-HCl was then added by syringe to the rotor centre, displacing an equal volume of CsCl from the edge. The rotor was accelerated to 40,000 rpm and run for 18 hours. At termination, the rotor was decelerated to 3000 rpm, and the gradient was displaced from the rotor edge by pumping Tris buffer to the rotor centre. Twenty millilitre fractions were manually

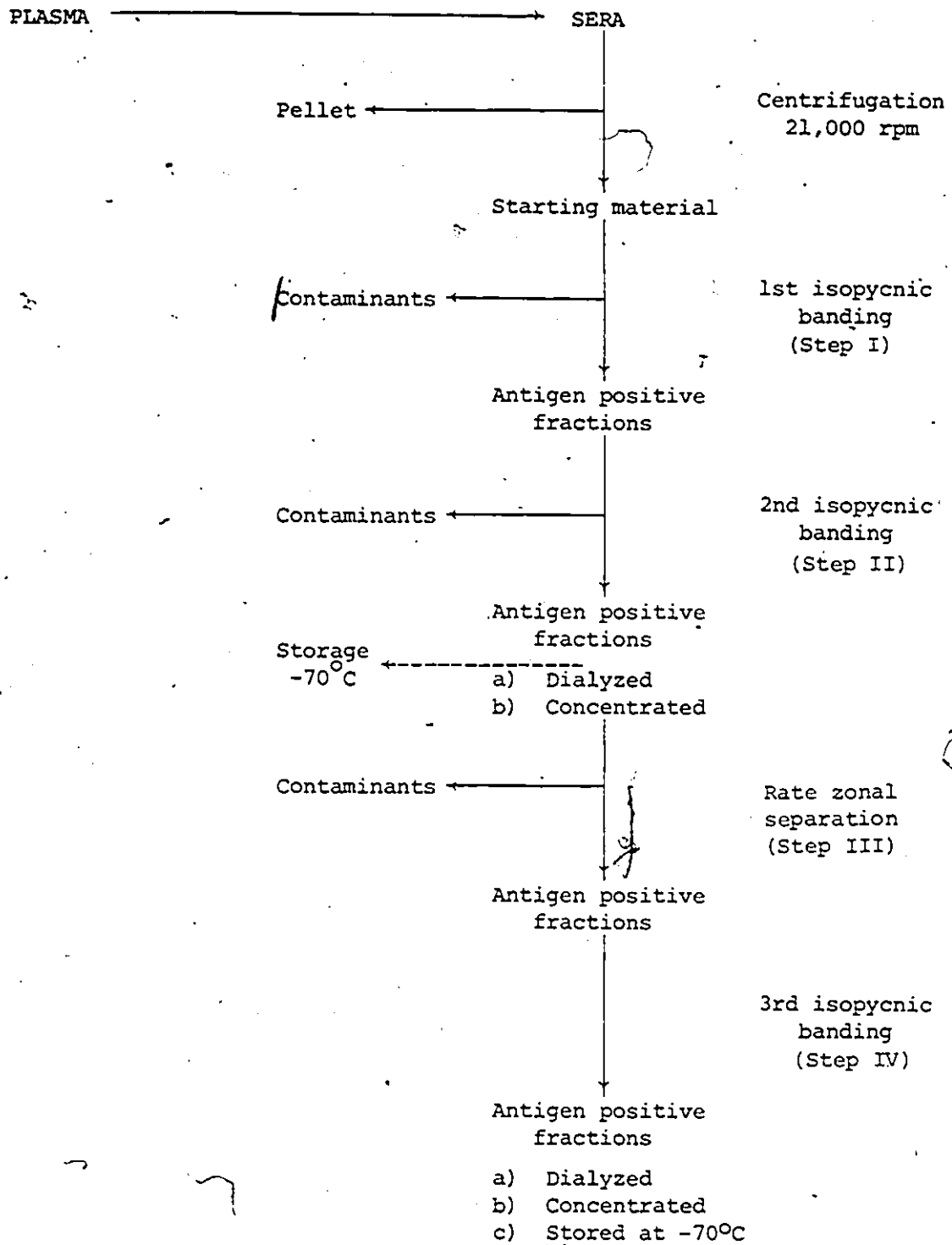


Figure 6:-- Large Volume Purification of HB_sAg

collected and assayed by RIA at dilutions of 10^0 , 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} .

- (2) Second isopycnic banding--Step 11. Antigen-positive fractions were pooled and adjusted to a density of 1.30 g/cm^3 by adding 1.60 g/cm^3 CsCl. A Ti-14 rotor spinning at 3000 rpm was sequentially edge-loaded with 110 ml of 1.10 g/cm^3 CsCl, 100 ml of 1.20 g/cm^3 CsCl, 185 ml of antigen-CsCl mixture at 1.30 g/cm^3 and 150 ml of 1.40 g/cm^3 CsCl to fill. The rotor was accelerated to 40,000 rpm for 18 hours. Decelerating the rotor to 3000 rpm terminated the run and the gradient was edge-unloaded, passing through a Gilford 2400 spectrophotometer at 280 nm. Twenty millilitre fractions were manually collected. Fractions positive for HB_sAg by RIA at dilutions previously described were pooled, dialyzed against PBS using an Amicon 65 cell with a Diaflo, XM-100A membrane (10 psi) and concentrated to 45 ml. At this stage, the antigen-positive sample could either be stored at -70°C or further purified. Antigen was designated CS2.

- (3) Rate zonal separation--Step III. A pre-cooled Ti-14 rotor spinning at 3000 rpm was edge-loaded with 410 ml of a continuous 10-30% w/w

sucrose gradient using an IEC model 3660 gradient former. This was followed by approximately 125 ml of 45% w/w sucrose to fill the rotor. Twenty-five millilitres of CS2 antigen was centre-loaded with a syringe followed by 50 ml of 0.01M Tris-HCl overlay, displacing approximately 75 ml of 45% w/w sucrose at the rotor edge. The rotor was accelerated to 40,000 rpm for 4 hours, decelerated to 3000 rpm and edge-unloaded as previously described in (1) and (2). Twenty millilitre fractions were manually collected following UV monitoring at 280 nm using an Oak Ridge 1 cm light path flow cell. Fractions positive for HB_sAg at 10⁻⁴ by RIA were pooled and diluted to 100 ml by 0.01M Tris-HCl.

- (4) Third isopycnic banding--Step IV. One hundred millilitres of 0.01M Tris, 100 ml of diluted antigen, 170 ml of 1.30 g/cm³ CsCl and 170 ml of 1.50 g/cm³ CsCl were sequentially edge-loaded into a spinning Ti-14 rotor. The rotor was accelerated to 40,000 rpm for 22 hours, decelerated to 3000 rpm and edge-unloaded as previously described. Twenty millilitre fractions were manually collected following UV scanning at 280 nm and assayed for HB_sAg by

RIA as previously described. Samples positive for HB_sAg were pooled, dialyzed against PBS in an Amicon 65 cell using an XM-100A membrane (10 psi), concentrated to 50 ml, dispensed in 1.0 ml aliquots and stored at -70°C.

e) Small volume purification:--This purification procedure was adapted from the large volume purification technique just described. Following conversion of plasma to serum and a 4 hour centrifugation at 21,000 rpm, HB_sAg-positive sera were centrifuged at 27,000 rpm for 24 hours in a fixed angle 30 rotor. The supernatant was decanted and the pellets resuspended in PBS. The supernatant was recentrifuged and the resulting pellets again resuspended in PBS. The pellet-PBS mixtures were pooled and adjusted to 10% of the starting volume with PBS. This material served as the starting sample for the 4-step purification procedure (figure 7). All centrifugations were done using an SW41 rotor in a Beckman L3-50 ultracentrifuge. Speed of centrifugation and temperature were 35,000 rpm and 5°C respectively.

(1) First isopycnic banding--Step I. Five millilitres of starting material were layered over 6 ml of 1.40 g/cm³ CsCl. The antigen was then overlaid with 1.5 ml of 0.01M Tris-HCl and centrifuged for 24 hours. Following centrifugation, 1 ml fractions were collected by bottom puncture and assayed for HB_sAg at

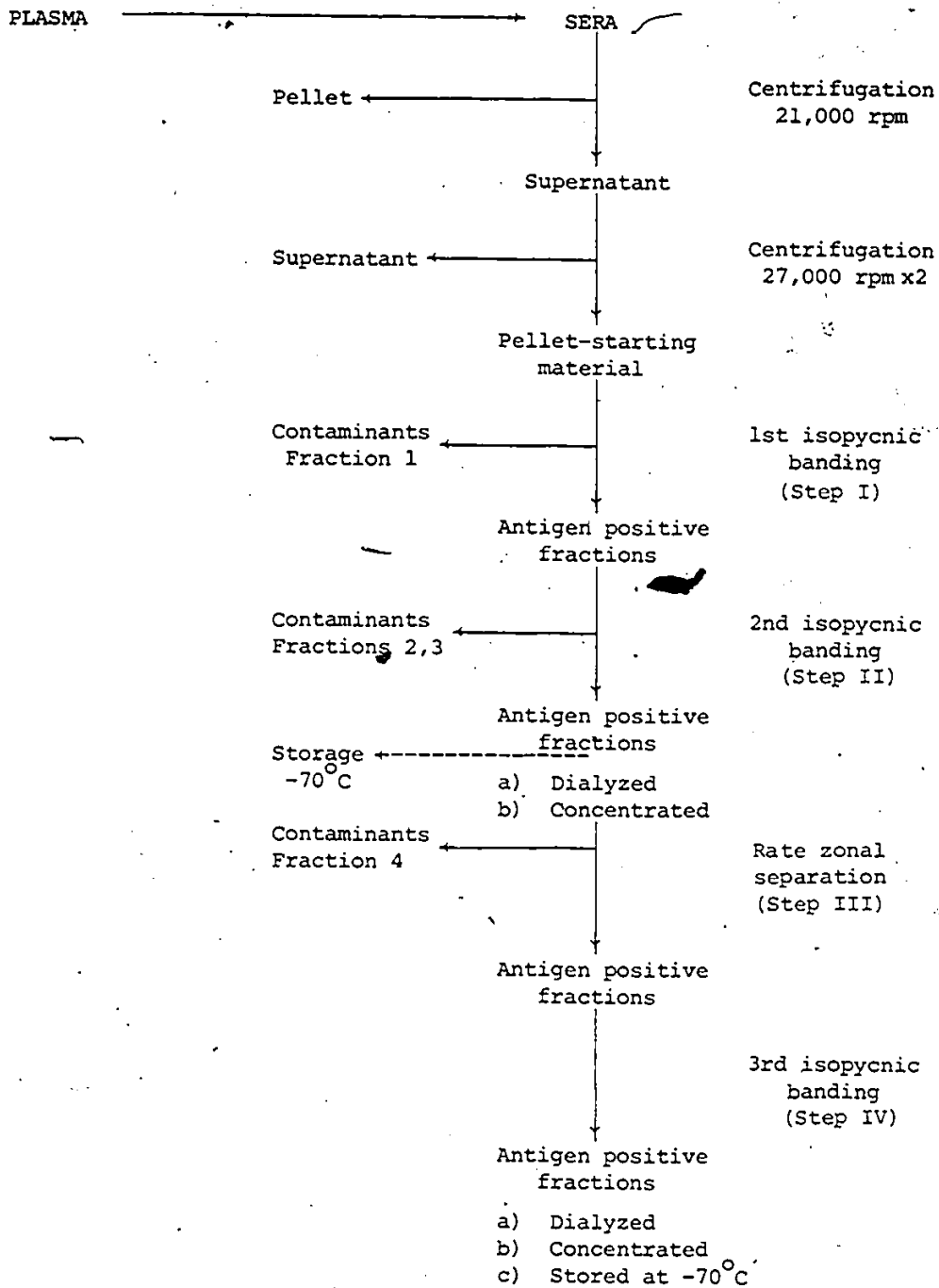


Figure 7:-- Small Volume Purification of HB_S Ag

dilutions of 1:8 to 1:512 by CIEP.

(2) Second isopycnic banding--Step II. Antigen-positive fractions were pooled and adjusted to a density of 1.30 g/cm^3 with 1.60 g/cm^3 CsCl. A four-step discontinuous gradient was formed by sequentially underlayering 2.0 ml of 1.10 g/cm^3 CsCl with 1.5 ml of 1.20 g/cm^3 CsCl, 5.0 ml of 1.30 g/cm^3 antigen-CsCl mixture and 4.0 ml of 1.40 g/cm^3 CsCl. The tubes were centrifuged for 24 hours and at termination, 0.6 ml fractions were collected by bottom puncture, UV scanned at 280 nm in a Hitachi spectrophotometer and assayed at dilutions of 1:8 to 1:512 for HB_sAg by CIEP. Antigen positive fractions were pooled, dialyzed in dialysis tubing against 200 volumes of PBS and concentrated in an Amicon 65 cell with a XM 100A membrane (10 psi). The antigen could either be stored at -70°C or further purified. It was designated CS2.

(3) Rate separation--Step III. A 7-35% continuous w/w sucrose gradient was formed in tubes by sequentially underlayering 1.5 ml of 7% w/w sucrose with equal volumes of 15%, 20%, 25%, 30% and 35% w/w sucrose and keeping it overnight at 4°C . After allowing for overnight

diffusion, the gradient was underlayered with 2.5 ml of 45% w/w sucrose to act as a cushion. One millilitre of the Step II or CS2 antigen was carefully layered onto the preformed gradient and the tubes centrifuged for 5 hours. Following centrifugation, 0.5 ml fractions were collected by bottom puncture, UV scanned at 280 nm and assayed for HB_sAg at 1:8 to 1:256 dilutions by CIEP.

- (4) Third isopycnic banding--Step IV. Fractions positive for HB_sAg were pooled and 4.0 ml were layered over a two-step discontinuous CsCl gradient formed by underlayering 3.8 ml of 1.30 g/cm³ with 3.2 ml of 1.50 g/cm³ CsCl. An overlay of 1.5 ml of PBS was carefully pipetted onto the antigen, and the tubes were centrifuged for 24 hours. The 0.75 ml fractions collected by bottom puncture were UV scanned at 280 nm and assayed for HB_sAg by CIEP as previously described. The antigen positive fractions were pooled, dialyzed against 200 volumes of PBS, concentrated in an Amicon 65 cell, dispensed in 1.0 ml aliquots and stored at -70°C.

f) Small volume purification following polyethylene glycol (PEG) precipitation:--HB_sAg was purified from serum

by a modification of a technique described by Cruceanu et al (1973). Antigen positive serum was adjusted to pH 4.6 with 2N HCl. PEG 6000 (Baker Chemical Co., Phillipsburg, N.J.) powder was added with continuous stirring to a concentration of 8%. The resulting precipitate was pelleted by centrifugation at 3500 rpm for 10 minutes and resuspended in PBS, pH 4.6 to 100 ml. This procedure was repeated 3 times, the final sediment being dissolved in 5 ml of PBS. The supernatants and the final sediment were assayed for HB_s Ag by CIEP. Antigen was purified by the small volume purification procedure described in e).

g) Density determinations:--The densities of the CsCl and sucrose fractions obtained from the three purification procedures described (d, e and f) were determined by a Hitachi refractometer and by relating refractive indices to densities from available tables.

h) Protein determinations:--Protein concentrations of the purified antigens were determined by using the Lowry technique. Antigen concentrations were expressed as µg/ml.

i) Electron microscopy:--Antigen positive fractions from Steps III and IV were studied. A drop of antigen (dialyzed against PBS) was placed on a formvar coated copper grid for 30 seconds. This was followed by a drop of 2% phosphotungstic acid, pH 7.2 for an additional minute. The excess fluid was blotted off with filter paper and the grid allowed to air dry. The grid was then examined in a Phillips 300 electron microscope.

j) Purity of Step IV antigen:--This antigen was tested for contaminants against rabbit anti-human serum (Hoechst Pharmaceuticals, Montreal, Quebec) by AGD and CIEP. In addition, 10 µg of Step IV antigen in complete Freund's adjuvant were inoculated into the foot pads of 6 guinea pigs. Two weeks later, each animal received a 10 µg antigen booster but with no adjuvant. Five millilitres of blood were taken by cardiac puncture immediately before the first inoculation of antigen and every week thereafter for 6 weeks. The guinea pig sera obtained on this weekly basis were assayed for both anti-HB_s and anti-human serum (anti-NHS) components by AGD.

C. Hepatitis B e antigen and antibody

1. Detection of HB_e Ag and anti-HB_e

HB_e Ag and anti-HB_e were tested for by a modification of an immunodiffusion technique described by Williams and LeBouvier (1976). Agarose A37 (Indubiose, Fisher Labs, Fairlawn, N.J.) was made up fresh (in 0.005M Tris-HCl-0.15M saline buffer, pH 7.6 containing 0.1% sodium azide) to a concentration of 0.4%. Dextran T500 (Pharmacia, Uppsala, Sweden) was added to a concentration of 0.5% to sharpen the lines (McAuliffe, personal communication). An uncoated 2.5° cm x 7.5 cm glass slide was covered with 1.3 ml of agarose A37 and allowed to harden overnight at 4°C. A six-shooter pattern (see figure 3) was cut in the hardened gel and 4.5 mm diameter agarose plugs were removed by gentle suction. There was an interwell distance of 3.5 mm

from well-edge to well-edge. The peripheral gel surrounding the wells was cut away to minimize outward diffusion of reactants. An e_1, e_2 antigen standard (courtesy Dr. M. Goldfield) was pipetted into the "A" wells while the four peripheral "S" wells were filled with HB_s Ag-positive samples. The central "a" well was filled with a known e_1, e_2 antibody (courtesy Dr. N. Nath and Dr. V. J. McAuliffe). All wells were filled 3 times within the first 2-3 hours and incubated in a moist chamber at room temperature for 48-72 hours. The slides were read and occasionally kept moist for up to 2 weeks at $4^\circ C$ for periodic rereading. Samples giving equivocal results were concentrated 5-10x and retested.

2. Populations studied

Sera from two institutions for the mentally retarded in Ontario (Rideau Regional Centre, Smith Falls and Huronia Regional Centre, Orillia) positive for HB_s Ag by CIEP were screened for HB_e Ag and anti- HB_e by the AGD technique. The residents of each institution were divided as to their diagnosis (DS vs OMR), age at admission and length of residence for evaluation. Controls matched to diagnosis, age at admission and length of residence but negative for HB_s Ag and anti HB_s were available from the Rideau Regional Centre and were also screened for HB_e Ag and anti- HB_e .

In addition, 100 consecutive sera from blood donors positive for HB_S Ag were obtained from the American Red Cross, Bethesda, Md. (courtesy of Dr. N. Nath). These were divided according to subtype and tested for HB_e Ag and anti-HB_e.

A third population was made up of 12 HB_S Ag-positive renal dialysis patients at the Ottawa General Hospital. These, too, were divided according to subtype and tested for HB_e Ag and anti-HB_e.

D. DNA polymerase assay (DPA)

1. Materials

(1) Solution 1--5% trichloroacetic acid (TCA)--a 1/10 dilution of a 50% TCA stock solution with distilled water.

(2) Whatman #3MM filter paper discs (Ø 2.4 mm). These were coded as to the samples that would be placed on them with a No. 2 lead pencil, wetted with solution 1 and dried in an oven at 37°C.

(3) Solution 2--stored at 4°C.

NP 40	1.0 ml
Distilled water	18.7 ml
B-mercaptoethanol	0.3 ml

(4) Reaction mixture--100 µl were prepared for each sample to be tested. Each component, except for the nucleotides, was stored

separately at 4°C.

1M Tris buffer, pH 7.5	17 μ l
1M MgCl ₂ .6H ₂ O.	3 μ l
2M NH ₄ Cl	6 μ l
Distilled Water	64 μ l
	<hr/>
	90 μ l
Nucleotides (2.5 μ l of each)	10 μ l
	<hr/>
	100 μ l

The nucleotides used were dATP, dCTP, dGTP (PL Biochemicals, Milwaukee, Wisc.) and ³H-TTP (New England Nuclear, Boston, Mass.). The 3 non-labelled nucleotides were made up as 0.02M stock solutions in distilled water and neutralized to a pH between 7-8 with a pinch of sodium bicarbonate (pH checked with litmus paper). These stock solutions were stored at -20°C and kept on ice when used in the reaction mixture. Two and one half microlitres of each of the stock solutions and 2.5 μ l of ³H-TTP were added for every sample to the 90 μ l of reaction mixture.

- (5) Solution 3--a 5% TCA solution in 0.1M sodium pyrophosphate (SPP) was prepared by dissolving 44.6 gm of SPP in 100 ml of 50% TCA and diluting the mixture 1:10 with distilled water.
- (6) Absolute ethanol.
- (7) Scintillation fluid.

PPO	5 gm
POPOP	0.3 gm
Toluene	1 litre

2. Method

This procedure was a modification of a technique first described by Kaplan et al (1973). Fourteen discs were used for the 7 negative controls (7 for 0 time, 7 for 180 minutes), 2 for the 2 positive controls, and 2 each for the samples to be tested. All samples, including the controls, were passed through a 0.45 μ millipore filter (MILLEX, Millipore Corp., Bedford, Mass.) with a 5 ml syringe and tested for sterility. Five microlitres of solution 2 were added to the bottom of an appropriate number of tubes using an Eppendorf pipette. This was followed by 25 μ l of each negative control, each positive control and each sample. These were mixed well and allowed to sit for a few minutes. Then, 100 μ l of the reaction mixture were added to all negative control tubes and mixed. Almost immediately thereafter, 50 μ l from each of the 7 tubes were spotted on appropriately marked discs, allowed to soak in and immersed in 5% TCA to stop the reaction. This constituted the 0 time for the negative controls. The remaining tubes all received 100 μ l of the reaction mixture, were covered with a parafilm and together with the remaining volumes of the negative controls, were incubated in a water bath at 37°C for 180 minutes.

After the incubation period, 50 μ l of each of the positive and negative controls were pipetted onto properly marked discs, allowed to absorb into the filter and placed into the 5% TCA solution. Fifty microlitres of each of the

antigen test samples were spotted on two appropriately marked filter papers, allowed to absorb into the filter discs and then submerged in the 5% TCA solution to stop the reaction. All discs were then transferred to a 4 litre beaker containing about 40 ml of solution 3 per disc. The beaker was placed on an electric shaker and the fluid and discs allowed to swirl at medium speed for approximately 60 minutes. It was important not to wash the discs too vigorously as this often caused them to disintegrate. The filters were taken out one by one and blotted on paper towels. This procedure of washing and blotting was repeated 2 more times. The filter discs were then washed in solution 1 for 60 minutes and blotted as before. If necessary, the discs could be left overnight in solution 3 at 4°C but then, had to be washed in fresh solution 3 for 30 minutes before washing in solution 1. Finally, the discs were dehydrated for about 5 minutes in absolute ethanol, blotted on paper towelling and dried in an incubator at 60°C. The discs were then transferred to scintillation vials containing 10 ml of scintillation fluid and counted in a Beckman LS-230 scintillation counter for 1 minute. The mean counts for the negative controls (180' - 0') and their standard deviation were then calculated and samples with counts per minute (CPM) equal to or greater than the negative mean plus 5 standard deviations were considered positive for DNA polymerase.

3. DNA polymerase specificity Assay (DPSA)

Samples positive for DNA polymerase were then tested for specificity. Fifty microlitres of test plasma or sera and 50 μ l of each of three negative and two positive controls were pipetted into each of a set of 3 tubes marked A, B and C. Five microlitres of guinea pig anti HB_s (NIH, "ad" antibody; HA titre 32,000) were added to tube A, 5 μ l of normal guinea pig serum to tube B and 5 μ l of PBS to tube C. These tubes were incubated overnight at 4°C and the following morning, 50 μ l of undiluted rabbit anti-guinea pig IgG (Flow Labs, Rockville, Md.) were added to each of the 3 tubes. All reagents were filtered through 0.45 μ Millex millipore filters before being added to the tubes. The tubes were incubated at 37°C for 90 minutes and centrifuged in an Eppendorf centrifuge for 2 minutes. Supernatants were carefully removed so as not to disturb the pellets and the DPSA was done on all the supernatants. Fifty microlitres of each supernatant were added to tubes containing 10 μ l of solution 2, followed by 200 μ l of the reaction mixture. Following 180 minutes incubation at 37°C, 100 μ l aliquots were pipetted onto appropriately labelled discs, allowed to maximally absorb, and then washed and counted as previously described. A reduction of counts in tube A of greater than or equal to 50% in HB_sAg-positive samples indicated specificity.

E. Hepatitis B core antigen
(HB_C Ag) and antibody

HB_C Ag was isolated from both liver tissue (post-mortem) and Dane particles circulating in human blood.

1. Liver

a) Microscopy:--A liver was obtained at necropsy from an immunosuppressed renal transplant recipient with chronic hepatitis B antigenemia. Five micron frozen sections were cut, formalin-fixed on glass slides for 10 minutes and washed with distilled water. The liver sections were stained directly with either fluorescein-conjugated anti-HB_C, anti-HB_S, or anti-IgG for 30 minutes and rinsed with distilled water. All fluorescein-conjugated antisera were passed through Sephadex G200 (Pharmacia, Uppsala, Sweden) Pasteur pipette columns immediately prior to use to remove all non-conjugated fluorescein. The sections were allowed to air dry, cover slipped and examined by phase contrast and polarizing microscopy to visualize hepatocytic architecture and the presence of HB_C Ag and/or HB_S Ag fluorescence.

b) Purification of HB_C Ag:--Core antigen was purified from liver by first isolating intact nuclei (a modification of a technique described by Chauveau et al, 1956) and secondly, by releasing HB_C Ag from these nuclei for further purification (Hirschman et al, 1974; Hirschman, personal communication). Liver samples of 30-50 grams were blotted dry with filter paper and finely minced with

scissors while on ice. The minced tissue was washed several times in cold saline (0.15M)-gentamicin (50 µg/ml)-NEM (0.005M) solution (Gentamicin-Schering Corp., Montreal, Quebec; NEM-Sigma Chemicals, St. Louis, Mo.) until the wash was relatively clear. The tissue was then washed several more times in cold saline-NEM without gentamicin. Following these washings, the liver was suspended in homogenizing medium (a mixture of 0.25M sucrose-0.0033M MgCl₂ - 0.005M NEM and 0.5M sucrose-MgCl₂-NEM) determined by the weight of the liver sample.

<u>Liver (grams)</u>	<u>0.25M Sucrose</u>	<u>0.5M Sucrose</u>
30	40	5
40	52.5	7.5
50	65	10

The liver was homogenized in a Virtis 45 homogenizer for 6 minutes at a medium setting (40) and the homogenate filtered through 4 layers of cheesecloth soaked in cold homogenizing medium. The homogenate was centrifuged at 5000 rpm for 10 minutes, the resulting pellet suspended in 10 ml of 0.25M sucrose-MgCl₂-NEM and further homogenized with 15-20 up and down strokes in a Dounce homogenizer with a tight fitting teflon pestle. The homogenate was underlayered with an equal volume of 0.34M sucrose-MgCl₂-NEM and centrifuged at 5000 rpm for 20 minutes. The resulting pellet was resuspended by homogenization in a Dounce homogenizer with a loose-fitting long-handled glass pestle in approximately 9 volumes of 2.4M sucrose-MgCl₂-NEM

and centrifuged at 18,000 rpm for 2 hours and 5°C. The pellet was resuspended in 10 ml of 0.25M sucrose-MgCl₂-NEM, washed in this solution by centrifugation 3 times at 8000 rpm for 15 minutes and examined by phase contrast microscopy. This pellet, which consisted primarily of nuclei, could either be stored in 0.25M sucrose at -20°C or further processed.

The nuclei were repelleted at 8000 rpm for 15 minutes, the sucrose discarded and the nuclei resuspended in 10 ml of sonication buffer (0.05M Tris-HCl, 0.04M KCl, 0.1% B-mercaptoethanol, 0.01M MgCl₂, 0.005M NEM, pH 7.5). The nuclei were again pelleted at 5000 rpm for 15 minutes and the pellet resuspended in 6 ml of sonication buffer. The suspension was poured into a metal tube (30cc capacity) and sonicated with a Bronwill III sonifier at 63 w/cm². A 30 second sonication was followed by a 30 second cooling period and this procedure was repeated 3 times for a total sonication time of 90 seconds. After each 30 second period, sonicated material was examined by phase contrast microscopy to determine the degree of nuclear disruption. Following sonication, the volume was adjusted upward to 10 ml with sonication buffer and centrifuged at 8000 rpm for 15 minutes. The pellet was discarded and the supernate was centrifuged at 18,000 rpm for 4 hours. The resulting pellet was resuspended in 0.05M Tris-HCl, pH 7.6 and adjusted to 1.30 g/cm³ with 1.60 g/cm³ CsCl. Five millilitres of the pellet suspension were underlayered with 4 ml

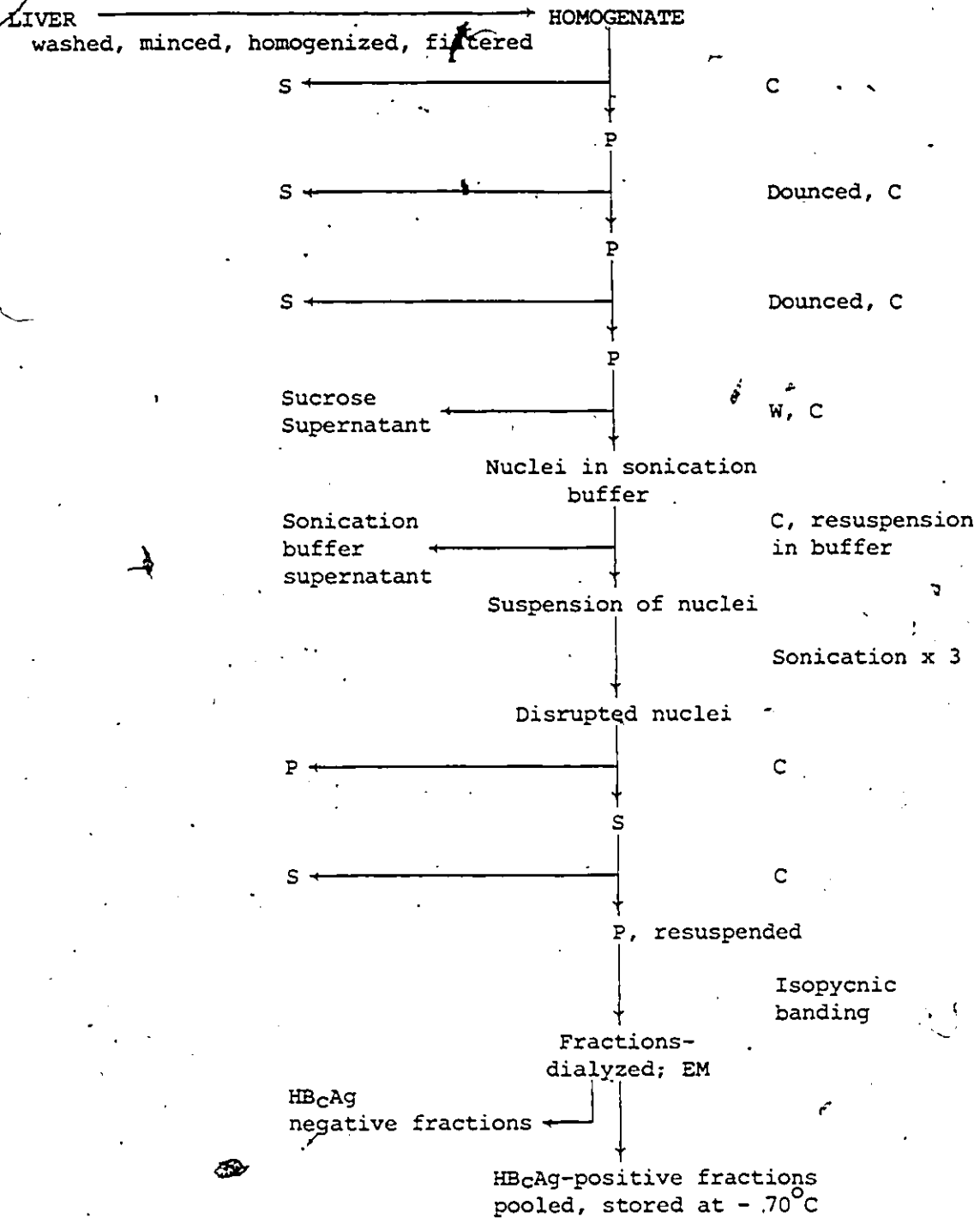
of 1.40 g/cm³ CsCl, overlaid with 1.5 ml of 1.20 g/cm³ CsCl and 2.0 ml of 1.10 g/cm³ CsCl and centrifuged at 35,000 rpm for 24 hours and 5°C in a Beckman L3-50 ultracentrifuge using an SW41 rotor. Fractions were collected from the tubes by bottom puncture and dialyzed against 100 volumes of PBS at 4°C. Fractions positive for cores by electron microscopy were pooled and stored at -70°C. The purification procedure is outlined in figure 8.

c) Electron microscopy:--All supernatants, pellets and gradient fractions were examined in a Phillips 300 EM on coated copper grids after staining with 2% PTA (phosphotungstic acid), pH 7.2.

2. Serum

a) Electron microscopy:--Pellets obtained from HB_sAg-positive serum after the 4 hour-21,000 rpm centrifugation described in section B 4c) were examined in a Phillips 300 EM after negative staining with 2% PTA, pH 7.2. Pellets found to contain more than 100 Dane particles/10 fields were selected for further purification.

b) Dane particle purification:--Dane-rich pellets were adjusted to a volume of 10 ml with PBS and layered over 20 mls of a 5-25% w/w continuous sucrose gradient. The sucrose gradient was underlayered with 5 ml of 35% w/w sucrose and centrifuged at 21,000 rpm for 4 hours using a fixed angle 30 rotor in an L3-50 ultracentrifuge. Two millilitre fractions were collected by bottom puncture,



KEY

- C--Centrifugation
- W--Wash
- S--Supernatant
- P--Pellet

Figure 8:-- Isolation of Cores from Liver Tissue

dialyzed against PBS and examined in an EM. Dane-rich fractions were pooled for additional purification. A rate separation of a 1 ml sample on a 7-35% w/w continuous sucrose gradient as described in section B 3e) (3), for small volume purification was carried out, and 0.5 ml fractions were collected by bottom puncture. These were UV scanned in a Hitachi spectrophotometer at 280 nm, tested undiluted by CIEP, dialyzed against 200 volumes of PBS at 4°C and examined in an EM.

c) Core extraction:--The Dane-rich fractions were pooled, diluted to 100 ml in 0.05M Tris-HCl and centrifuged for 6 hours at 21,000 rpm using a fixed angle 30 rotor in an L3-50 ultracentrifuge. The pellets were resuspended in 2 ml of 0.05M Tris-HCl containing 50 µl NP40 (Shell Oil Co., Montreal, Quebec) and 15 µl B-mercaptoethanol (Eastman Kodak, Rochester, N.Y.) and allowed to incubate for 1 hour at room temperature with occasional shaking. One millilitre of this antigen-buffer mixture was layered on top of a continuous 10-45% w/w sucrose gradient on a 60% w/w sucrose cushion and centrifuged for 5 hours at 35,000 rpm and 5°C using an SW41 rotor in an L3-50 ultracentrifuge. One half millilitre fractions were collected by bottom puncture, dialyzed against PBS and examined for core antigen by standard and immune electron microscopy. The procedures outlined in b) and c) are schematically shown in figure 9.

d) Immune electron microscopy (IEM):--Guinea pig anti-HB_s and guinea pig anti-HB_c (courtesy of Dr. J. Hoofnagle)

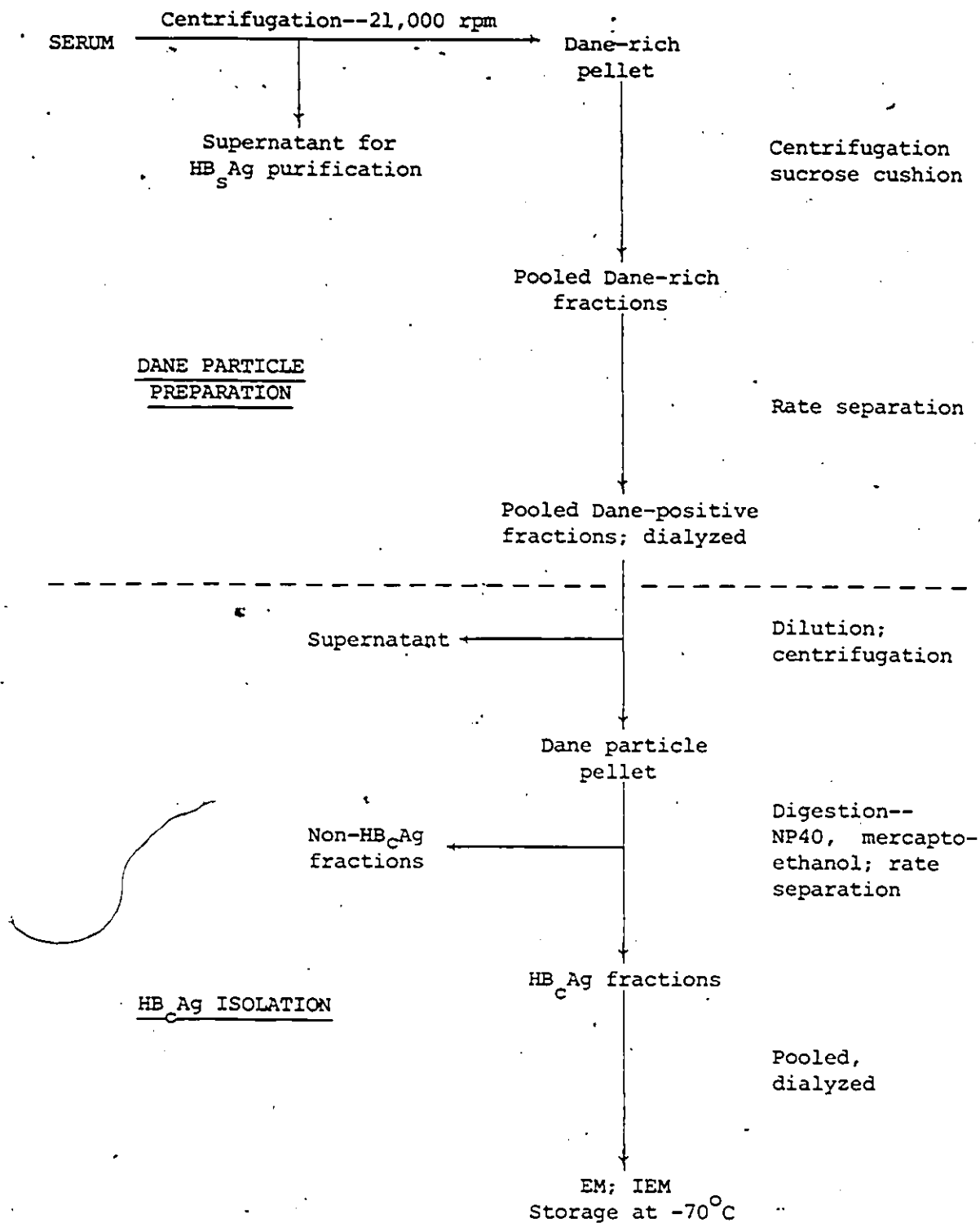


Figure 9:-- Purification of Cores from Serum

were used to test the identity of the extracted core antigen. Twenty-five microlitres of either antiserum were mixed with 75 μ l of the core preparation and allowed to incubate overnight at 4°C. A drop of the antigen-antiserum mixture was spotted on a coated copper grid. After 20-30 seconds, a drop of 2% PTA, pH 7.2 was added to the grid for 1 minute. The excess fluid was blotted off the grid with absorbent paper and the grid allowed to air dry. The grid was then examined in the EM.

e) Anti-HB_c detection:--Anti-HB_c was detected with a commercially available RIA kit manufactured by Abbott Labs (N. Chicago, Ill.)

II. Cell Mediated Immunity

Two sets of experiments were designed to study the cell mediated immune response to HB_sAg. In the first, two lymphocyte stimulation assays--whole blood cultures and lymphocyte cultures--were compared and then contrasted with a leukocyte migration inhibition assay in agarose. The second set of experiments looked at lymphocyte stimulation using different HB_sAg preparations and concentrations as well as serum byproducts obtained from the purification procedure (see figure 6). These are outlined in Table II.

A. Populations Tested

In experiment 1, 6 post-hepatitis B donors with proven HB_sAg-positive hepatitis, and who became negative for

TABLE II

EXPERIMENTAL PROTOCOL FOR CMI STUDIES

Group	Whole blood cultures	Lymphocyte cultures	Group	Lymphocyte cultures in pooled AB negative sera
<p>Experiment I: Matched post-hepatitis B donors with HB_sAg/anti-HB_s negative controls.</p>				
A	Control	Control	A	Control
B	PHA - 10 µg	PHA - 10 µg	B	PHA - 10 µg
C	HB _s Ag-2 µg	HB _s Ag-2 µg	C	HB _s Ag-2 µg
D	5 µg	5 µg	D	5 µg
E	25 µg	25 µg	E	25 µg
<p>Migration Inhibition</p>				
<p>Controls</p>				
<p>HB_sAg- 40 µg/ml</p>				
<p>- 80 µg/ml</p>				
<p>Experiment II: Matched post-hepatitis B donors with HB_sAg/anti-HB_s negative controls.</p>				
<p>Lymphocyte cultures in pooled AB negative sera</p>				
<p>HB_sAg vaccine 2 µg</p>				
<p>5 µg</p>				
<p>25 µg</p>				
<p>Fraction 1 (0.1 ml)</p>				
<p>2 (0.1 ml)</p>				
<p>3 (0.1 ml)</p>				
<p>4 (0.1 ml)</p>				
<p>Fraction 1' (0.1 ml)</p>				
<p>2' (0.1 ml)</p>				
<p>3' (0.1 ml)</p>				
<p>4' (0.1 ml)</p>				

antigen by CIEP and RIA, with or without anti-HB_S were selected for lymphocyte stimulation and migration inhibition testing. These were matched by 6 negative controls (no exposure to HB_SAg and anti-HB_S-negative. Experiment II compared 10 post-hepatitis B and/or anti-HB_S positive individuals with 10 healthy controls with no previous exposure to HB_SAg and who were anti-HB_S negative.

B. Lymphocyte Stimulation

1. Parameters

Various factors, including serum supplements added to the culture medium (autologous, homologous-pooled AB negative sera, and heterologous-fetal bovine sera) and pH were studied to determine whether they had any effect on lymphocyte stimulation. In addition, optimal incubation periods for the various antigens and mitogens used were also determined.

2. Whole blood technique.

This method was adapted from a procedure described by Pauly et al (1973).

a) Lymphocyte culture preparation:--Five millilitres of blood were collected by venipuncture and transferred immediately into a sterile container containing sodium-free heparin (Nutritional Biochemical, Cleveland, Ohio) to a final concentration of 10 units heparin per millilitre of blood. Ninety-five millilitres of RPMI 1640 (Flow Labs, Rockville, Md.) were made up as follows:

9.0 ml 10xRPMI 1640
2.5 ml of 1M HEPES buffer
1.0 ml of 200mM L-glutamine
0.75 ml of 7.5% sodium bicarbonate
1.0 ml of Pen/Strep (10,000U penicillin-
10 mgm streptomycin)
80.75 ml of sterile distilled water.

The medium was filtered through a Nalgene 0.20 μ filter (Sybron Corporation, Rochester, N.Y.) and added to the blood-heparin mixture to produce a 1:20 whole blood suspension. The diluted whole blood was continuously mixed at low speed on a magnetic stirrer and 3.0 ml aliquots were pipetted aseptically in 16x125 mm disposable plastic culture tubes (Falcon Plastic, Oxnard, Calif.). Cultures were tightly capped, placed upright in a rack and incubated at 37°C.

b) Lymphocyte stimulants:--All stimulants were prepared in unsupplemented RPMI 1640 (1x) to the desired concentrations and dispensed in 0.4 ml amounts to each culture tube.

- (1) Antigen. Purified HB_sAg/adw was diluted to 2, 5 and 25 μ g protein/0.4 ml RPMI 1640 and dispensed in triplicate group C, D and E cultures respectively.
- (2) Mitogen. Purified Wellcome phytohaemagglutinin (PHA; Warner Chilcott, Toronto, Ont.) was diluted to 25 μ g/ml and 0.4 ml (10 μ g) was dispensed to triplicate group B cultures.

(3) Control. All 6 group A cultures received 0.4 ml of unsupplemented 1xRPMI 1640.

c) Culture conditions:--All group B and 3 of the group A cultures were incubated for 2 days whereas the remaining group A and all group C, D and E cultures were incubated for 6 days. Each day, pH and viability (trypan blue exclusion) were determined to insure optimal culture growth conditions.

d) Lymphocyte stimulation measurement:--One day prior to harvest (either day 1 or day 5, depending on the group), 1.0 μ Ci of tritiated thymidine (3 H-T; specific activity 2.0 Ci/m mole; Amersham Corp., Oakville, Ont.) in 0.2 ml of unsupplemented 1xRPMI 1640 was added to each culture. The cultures were agitated on a Vortex mixer and incubated for an additional 24 hours. At harvest, the cells were resuspended and transferred to a 12 ml conical glass centrifuge tube. The culture tubes were rinsed several times with cold 3% acetic acid and the washings added to the glass tubes. The cells were pelleted by centrifugation at 2000 rpm for 8 minutes, the supernatant aspirated with a glass pipette connected by rubber tubing to a double flask water tap, resuspended in 12 ml of cold 3% acetic acid and repelleted. This procedure was repeated twice. Following the final pelleting and aspiration, one drop of 30% hydrogen peroxide was added to each cell button with a pipette to decolor residual red cells. The uncapped

tubes were then placed in a prewarmed 85°C oven for 20 minutes to enhance oxidation and to decrease the moisture in the tubes. The cell buttons were dissolved in 0.6 ml of NCS tissue solubilizer (Amersham Corp.) for 1 minute. Five millilitres of scintillation fluid (5 gram PPO/litre toluene) were added to each glass tube and mixed on a Vortex mixer. An additional 5 ml of scintillation fluid were added to each tube and the contents of each tube transferred to appropriately labelled scintillation vials (New England Nuclear, Boston, Mass.). After allowing for the vials to dark adapt overnight, they were counted for 2 minutes in a Beckman LS 230 liquid scintillation counter and the results expressed as CPM minus background (the latter usually between 30-70 cpm). A stimulation index was calculated from the formula

$$\text{S.I.} = \frac{\text{Mean of the test cultures}}{\text{Mean of the control cultures}}$$

3. Lymphocyte cultures

This is an adaptation of an earlier technique described by Bloom and Glade (1971).

a) Lymphocyte separation:--Sixty millilitres of blood were collected by venipuncture and immediately transferred to a sterile flask containing 600 units sodium-free heparin. The blood was gently swirled in the flask to insure thorough mixing before an equal volume of sterile 0.85M saline was added. The diluted blood was placed on a

magnetic stirrer at low speed for continuous mixing. Four millilitres of lymphocyte separation medium (LSM-Bionetics, Kensington, Md.) were pipetted into sterile glass tubes and carefully overlaid with 8 mls of diluted blood, insuring that an interface remained between the blood and LSM. The tubes were sealed with parafilm and centrifuged at 800 rpm for 30 minutes. The serum was pipetted off and discarded and the cellular layer at the interface harvested. The cells were transferred to a glass centrifuge tube and washed two times in Hanks Balanced Salt Solution (HBSS) containing 50 µg gentamicin/ml and a third time in HBSS alone. Following examination by light microscopy (>90% lymphocytes), the cells were adjusted to 10^6 /ml in prewarmed (37°C) RPMI 1640 freshly prepared and pre-filtered through a 0.20 µ Nalgene filter.

12 ml of 10xRPMI 1640
3.0 ml of 1M HEPES buffer
1.5 ml of 7.5% sodium bicarbonate
1.2 ml of 200 mM L glutamine
1.3 ml of pen/strep (10,000U penicillin/10 mgm streptomycin)
12 ml of pooled human AB negative sera* (or FBS, Exp. 1)
*(4 sera--heat inactivated; Cdn. Red Cross, Ottawa)
89 ml of sterile distilled water
1N sodium hydroxide to adjust pH to 7.4

One millilitre of medium containing 10^6 cells was pipetted into each plastic 16x125 mm culture tube, tightly capped and incubated in an upright position at 37°C.

b) Lymphocyte stimulants:--Stimulants were added at desired concentration in 0.2 ml aliquots by either diluting in unsupplemented 1xRPMI 1640 or concentrating in

an Amicon 65 cell with an XM 100A membrane.

- (1) Antigens. Groups C, D and E received 2, 5 and 25 μg HB_sAg/adw in 0.2 ml RPMI 1640 respectively. Groups F, G and H received like amounts of the Bureau of Biologics HB_sAg/adw vaccine (courtesy Dr. J. L. Gerin). The vaccine, which was a formalin inactivated HB_sAg preparation stabilized with 0.1% human serum albumin, was extensively dialyzed against PBS prior to use to remove the inactivating agent. Groups I, J, K and L received 0.1 ml of 1:8 dilutions of serum contaminants 1, 2, 3 and 4 recovered during the small volume purification procedure (see figure 7) while groups M, N, O and P received 0.2 ml of like fractions obtained following purification of an HB_sAg-negative serum (1', 2', 3' and 4'). All cultures were set up in triplicate.
- (2) Mitogen. Purified PHA was diluted to 50 $\mu\text{g}/\text{ml}$ and 0.2 ml (10 μg) was added to the triplicate B cultures.
- (3) Control. All 6 group A cultures received 0.2 ml of unsupplemented 1xRPMI 1640.

c) Culture conditions:--These were identical to those previously described for the whole blood technique.

d) Lymphocyte stimulation measurement:--One day prior to harvest (day 2 for group B and 3/6 group A; day 6 for the remaining groups), 1.0 μ Ci of $^3\text{H-T}$ in 0.2 ml RPMI 1640 was added to each culture. The cultures were resuspended on a Vortex mixer and allowed to incubate for an additional 24 hours. The cells were then resuspended and transferred to 12 ml glass centrifuge tubes. Each culture tube was washed several times with cold 0.85N NaCl and the washings added to the glass tubes. These tubes were centrifuged at 2000 rpm for 8 minutes and the supernatant aspirated as previously described. The cells were rewashed in cold saline, followed by two washes in cold 5% trichloroacetic acid. The pellets were dehydrated in an 85°C oven for 1 hour before 0.5 ml NCS solubilizer was added. One minute later, 5 ml of scintillation fluid was added to each tube and mixed thoroughly. An additional 5 ml of scintillation fluid was added to each tube and the contents transferred to appropriately labelled scintillation vials. The vials were allowed to dark adapt overnight and the samples were counted in an LS230 liquid scintillation counter for 2 minutes. The results were expressed as counts per minute and an S.I. was calculated for each sample (mean of triplicate cultures).

C. Migration Inhibition

1. Preparation of agarose

The Clausen (1971) agarose migration inhibition technique was used. A 50 ml 2% solution of agarose A45

was prepared and kept at 50°C in a water bath. This was made up to 100 ml by adding 10 ml of 10xTC medium 199 (GIBCO, Grand Island, N.Y.), 10 ml of heat inactivated horse serum (Flow Labs, Rockville, Md.), 1 ml of penicillin-streptomycin (10,000U penicillin, 10 mgm streptomycin), 1 ml of 7.5% sodium bicarbonate and 28 ml of sterile distilled water. This produced a 1% agarose solution containing single strength TC-199, 10% horse serum and 100 units penicillin-100 µg streptomycin/ml.

The agarose was then dispensed in 7.5 ml aliquots into 60x15 mm plastic petri dishes (Falcon Plastics, Oxnard, Calif.). The dishes were allowed to solidify and were incubated at 37°C in a humidified atmosphere containing 2% CO₂. Immediately prior to the addition of the preincubated leukocytes, 2.5 mm Ø wells were cut in the agarose using a stainless steel punch.

2. Preparation of leukocytes.

Thirty millilitres of blood were collected by venipuncture and transferred immediately to a sterile glass tube containing 300 units of sodium-free heparin and 6 ml of 6% dextran (m.w. 234,000, Sigma Chemicals, St. Louis, Mo.) in 0.85N saline. The tube was inverted several times to allow for thorough mixing and incubated at a 30° angle for 45 minutes at 37°C. This was followed by an additional 30 minute incubation period in an upright position. The leukocyte-rich fraction was carefully pipetted off into 4

12 ml glass centrifuge tubes and spun for 15 minutes at 1000 rpm. The supernatant was aspirated off and the tubes filled with 0.85% ammonium chloride to lyse the residual red cells. This wash was followed by three washes in HBSS containing 100 units penicillin-100 µg streptomycin/ml. After the final wash, single strength TC199 containing 10% heat-inactivated horse serum was added to the cells to a concentration of 2.4×10^8 cells/ml.

3. Addition of test reagents

Ten microliters of HB_sAg/adw in concentrations of 40 µg/ml and 80 µg/ml were added to 90 µl of leukocytes and incubated for 1 hour at 37°C. Controls consisted of 10 µl of single strength TC199 and 90 µl of leukocytes. These, too, were incubated for 1 hour. Following incubation, 7 µl aliquots of cells were pipetted into 8 freshly cut wells (approximately 1.5×10^6 cells/well) with a Hamilton syringe and incubated for 20 hours at 37°C in a humidified 2% CO₂ incubator.

4. Migration index

The plates were magnified by projection and the areas of migration measured by planimetry. The well area was not included in the calculation. A migration index (M.I.) was calculated from the formula

$$\text{M.I.} = \frac{\text{Mean of HB}_s\text{Ag incubated cells}}{\text{Mean of TC199 incubated cells}}$$

5. Identification of migrating cells

Following planimetry, the plates were placed on a 100°C water bath and the agarose allowed to soften. The plates were then filled with n-butanol for 30 minutes to fix the cells to the plate, the agarose carefully floated off and the plates stained with Wright's stain.

RESULTS

I. Antigen Studies

A. Hepatitis B surface antigen (HB_s Ag) and antibody (anti-HB_s)

1. Comparison of RIA, AGD and CIEP for HB_s Ag detection in serum (Table III)

a) RIA:--All 20 sera from the American Red Cross (ARC) HB_s Ag-positive panel were reconfirmed as positives. A serum was considered to be HB_s Ag-positive when the ratio of the test serum (P) to the negative control (N)--the P/N ratio--was 2 or greater. The ratios of the 20 sera were between 2.4 and 135.6.

b) AGD:--Only 14 of the 20 sera positive by RIA were positive by this technique. The resultant precipitating lines were scored 1+ - 4+ depending on their thickness, the thickness being somewhat indicative of the strength of the unknown antigen. The corresponding RIA P/N ratios of these 14 sera were 12.2 and greater.

c) CIEP:--Sixteen sera were HB_s Ag-positive by this technique. Antigen strength was scored 1+ - 4+, depending on the thickness of the precipitating line. Only sera with an RIA P/N ratio of 8.9 or greater were detectable.

TABLE III

COMPARISON OF RIA, AGD AND CIEP FOR THE
DETECTION OF HB_S Ag

Technique	HB _S Ag-positive panel (ARC)			HB _S Ag-negative panel (ARC)		
	No. positive	% positive	P/N (RIA)	No. positive	% positive	P/N (RIA)
RIA	20	100	2.4-135.6	0	0	<2
AGD	14	70	12.2-135.6 *	0	0	NT
CIEP	16	80	8.9-135.6 *	0	0	NT

ARC: American Red Cross

NT: Not tested

* The corresponding P/N ratios at which sera positive for HB_S Ag by RIA could be detected by AGD or CIEP.

None of the 20 sera from the HB_sAg-negative panel were found to be positive by any of these three techniques.

2. Comparison of HA and RIA for detecting anti-HB_s in serum (Table IV)

All sera from the ARC anti-HB_s-positive panel were reconfirmed as positives by these techniques. In addition, 1 of the 20 sera negative by RIA was anti-HB_s-positive by HA to a titre of 1:4. Retesting of this serum by RIA and HA reproduced the results shown in table IV.

3. Detection of HB_sAg and anti-HB_s in consecutive sera collected from an institutionalized population (Table V)

Of the 195 consecutive sera collected from the Down's syndrome (DS) population during a 4 year period, 70 were positive for HB_sAg by RIA and 66 were positive by CIEP (approximately 36% and 34% respectively). Antigenemia in the other mentally retarded (OMR) patients was approximately 12% by the two techniques.

The prevalence of anti-HB_s by the HA technique was comparable in the two populations. Sera negative for HB_sAg and anti-HB_s were more common in the OMR group.

No attempt was made to group these populations by age, sex, age at admission or length of residence.

4. Subtype determinations

Three AGD subtype patterns are demonstrated in figure 10. In each, the "A" wells were filled with

TABLE IV

COMPARISON OF HA AND RIA FOR

ANTI-HB_s DETECTION

Technique	Anti-HB _s -positive panel		Anti-HB _s -negative panel	
	No. positive	% positive	No. positive	% positive
HA	20	100	1	5
RIA	20	100	0	0

TABLE V
 PREVALENCE OF HB_SAg AND ANTI-HB_S IN CONSECUTIVE SERA
 COLLECTED FROM AN INSTITUTIONALIZED POPULATION

Population	No.	HB _S Ag-positive		Anti-HB _S -positive		HB _S Ag/anti-HB _S -negative	
		RIA (%)	CIEP (%)	HA (%)	RIA (%)	CIEP (%)	HA (%)
Down's syndrome (DS)	195	70 (35.9)	66 (33.8)	102 (52.3)	23 (11.8)	27 (13.9)	
Other mentally retarded (OMR)	2400	282 (11.8)	269 (11.2)	1276 (53.2)	842 (35.0)	855 (35.6)	

HB_sAg/ad, the "a" wells with HB_sAg/anti-ad and the "S" wells with subtype-unknown HB_sAg-positive sera for subtyping.

In figure 10a, the "S" wells showed identity to the "A" wells and to each other (lack of spur formation). The subtypes of the unknowns were identical to the known standards and in this example were all HB_sAg/ad.

In figure 10b, the 8 o'clock "S" well was only partially identical to the 6 and 10 o'clock "A" and "S" wells respectively as seen by the spur formation. The group specific determinant a was common to all peripheral wells but the type specific d determinant was not common to the 8 o'clock serum. This antigen was an HB_sAg/ay subtype.

The third pattern showed identity between the "S" wells (same subtype) but lack of identity with the "A" wells (spur formation). All wells had partial identity through the group specific a marker but the type specific d marker was absent from the "S" wells. These sera showed complete identity when tested against HB_sAg/ay and HB_sAg/anti-ay standards.

Sera collected from four distinct HB_sAg-positive populations were compared as to subtype prevalence. In 3 of the 4 populations, HB_sAg/ad was the predominant subtype (Table VI) whereas only in the acutely ill hepatitis B positive patients was ay predominant. Two sera in the blood donor population were untypeable, even after serum concentration (5x) and centrifugation at 3500 rpm to

Figures 10 a-c: Subtype Determination

- a. Complete identity of the unknown sera with a known subtype, in this case HB_sAg/ad.
- b. Partial identity of the serum in the 8 o'clock well with the known antigen subtype and the unknown sera in the three other peripheral wells. Partial identity is indicated by spur formation. The 2, 4 and 10 o'clock wells are HB_sAg/ad while the 8 o'clock well is HB_sAg/ay.
- c. Partial identity of the four unknown samples with a known antigen subtype, as evidenced by spur formation. Partial identity is with the "a" determinant only. The four unknown sera are HB_sAg/ay.

Figures 10 a-c: Subtype Determination

Fig. 10a

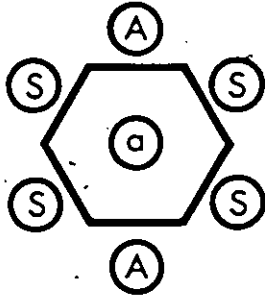


Fig. 10b

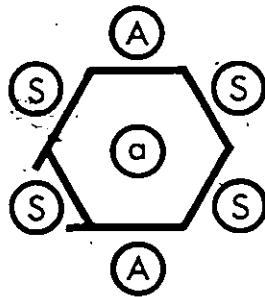
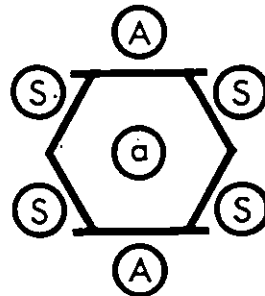


Fig. 10c



KEY

STANDARDS

A - KNOWN ANTIGEN
SUB TYPE

a - KNOWN ANTIBODY
SUB TYPE

TEST SERUM

S - UNKNOWN SUB TYPE

TABLE VI

SUBTYPES IN FOUR HB_s Ag-POSITIVE POPULATIONS

Population	No. of samples subtyped	Subtypes		
		ad ₁	ay	Untype- able
Blood donors (BD)	100	63	35	2
Other mentally retarded, Down's syndrome (OMR, DS)	100	100	0	0
Acute hepatitis B	100	2	98	0
Renal dialysis (RD)	12	12	0	0

remove debris.

Statistical analysis of the 4 groups revealed that these differences were significant ($p < 0.01$).

5. HB_sAg purification

a) Large volume purification:--This purification scheme for spherical 22 nm HB_sAg particles involved a series of 3 isopycnic bandings in CsCl and one sucrose rate zonal centrifugation. After the first isopycnic banding (CS1), antigenic activity by RIA at 10^{-4} dilution was recovered in fractions 7 to 10. Lower dilutions produced positive P/N ratios in almost all fractions, thereby making it difficult to select the appropriate fractions for further purification (figure 11a, b). Occasionally, at lower dilutions (10^{-1} , 10^{-2}), the antigen was excessive and a prozone effect resulted. This could be overcome if higher dilutions were measured by RIA (figure 12).

Fractions 7 to 10 were pooled, adjusted to a density of 1.30 g/cm^3 with CsCl and rebanded in a discontinuous CsCl gradient (see Materials and Methods). This second isopycnic banding (CS2) permitted the 22 nm HB_sAg particles to float free from the bulk of the lower molecular weight and higher serum density contaminants (figure 13). RIA activity at 10^{-4} dilution coincided completely with the 280 nm optical density peak. The CsCl buoyant density of the most reactive RIA fraction was 1.203 g/cm^3 (figures 13, 14). Electron microscopic examination of the 10^{-4} RIA-positive fractions revealed predominantly 22 nm

Figure 11 a and b: The first isopycnic banding from a large volume purification in a rate zonal rotor:

a. RIA P/N ratios at dilutions 10^0 , 10^{-1} and 10^{-2} .

b. RIA P/N ratios at dilutions 10^0 , 10^{-3} and 10^{-4} .

St. S. - Starting sample

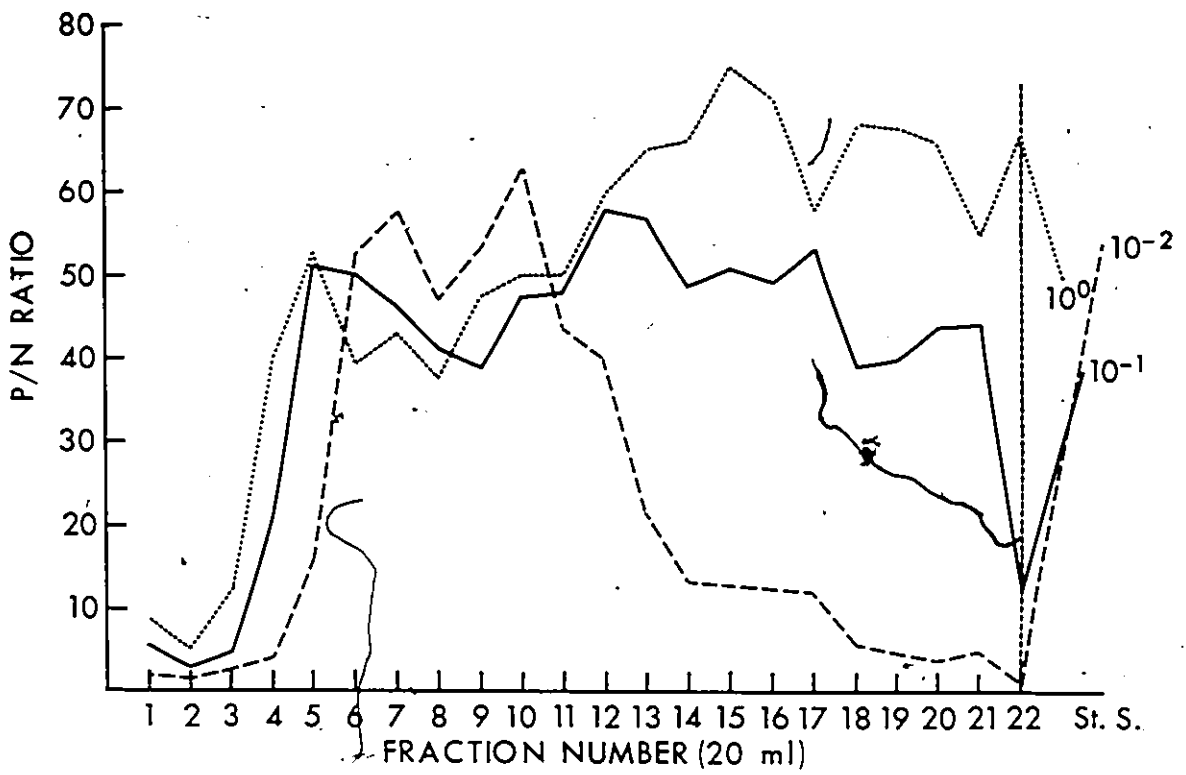


Figure 11a.

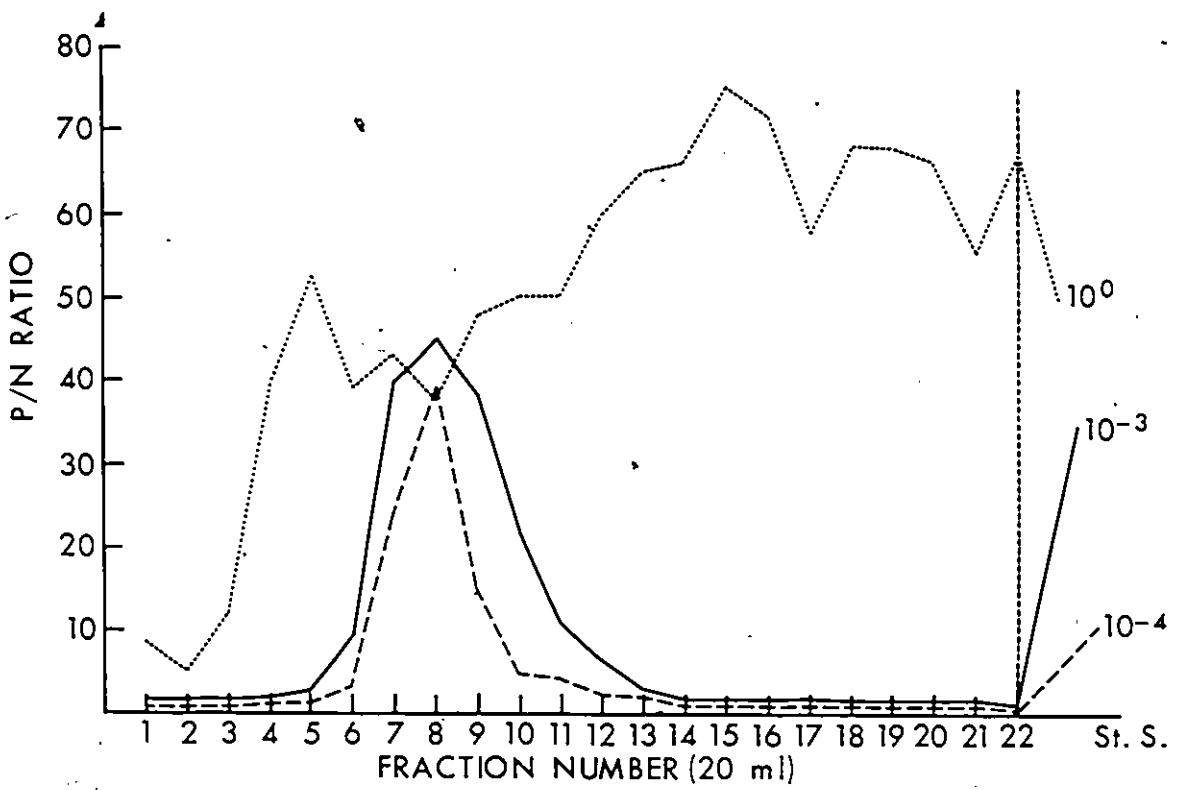


Figure 11b.

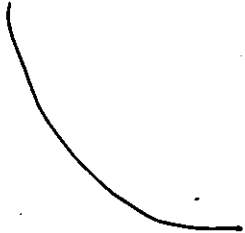


Figure 12: Prozone effect at a dilution of 10^{-2} when measured by RIA.

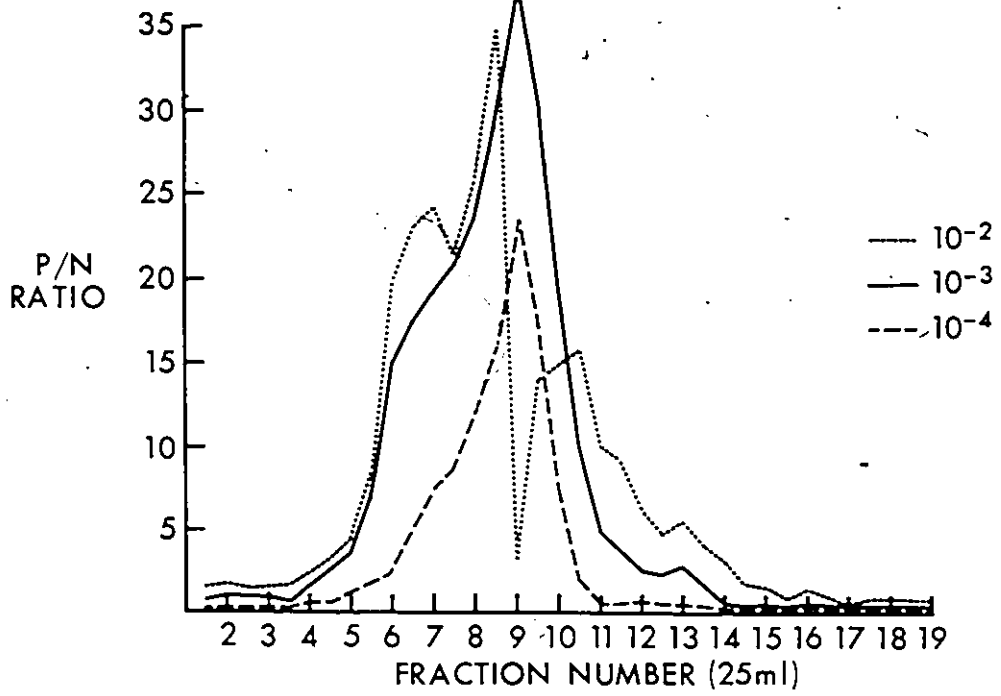


Figure 12

Figures 13 and 14

13. Second isopycnic banding in CsCl from the large volume purification procedure. Peak RIA activity coincided with a CsCl density of 1.203 g/cm^3 .
14. Optical density determination at 280 nm of the second isopycnic banding in CsCl. The second peak coincides with RIA activity

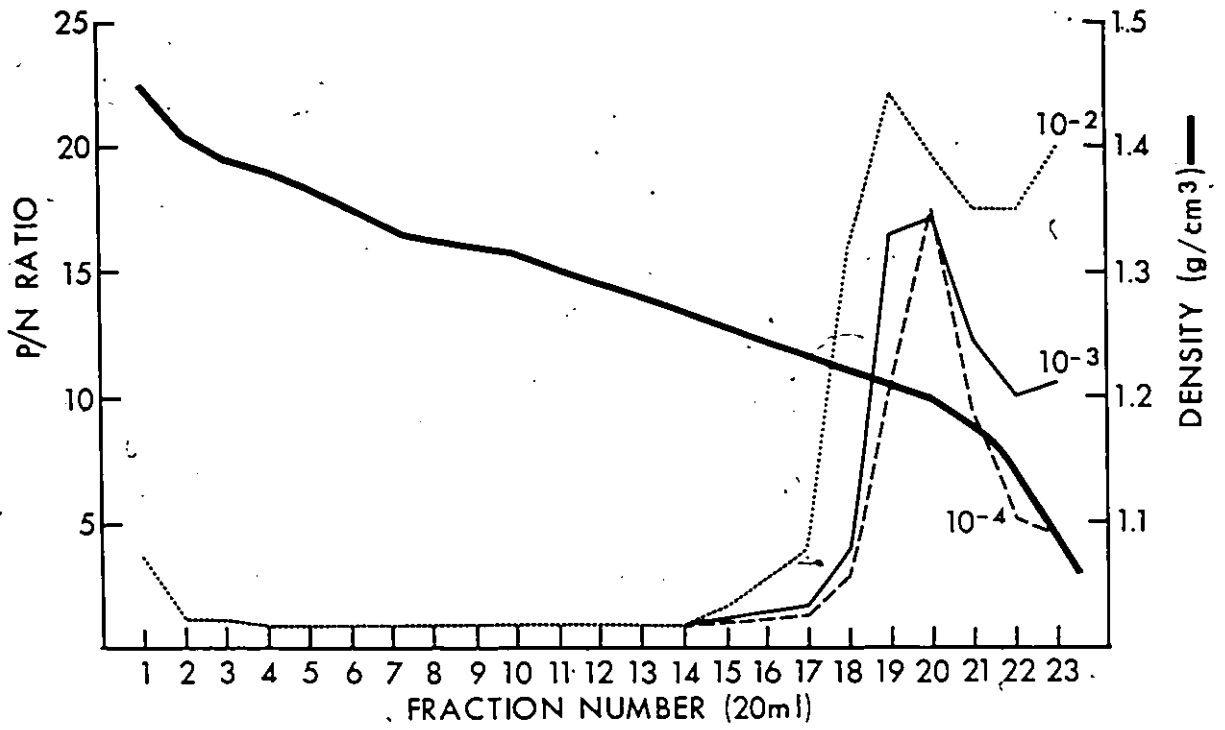


Figure 13 :

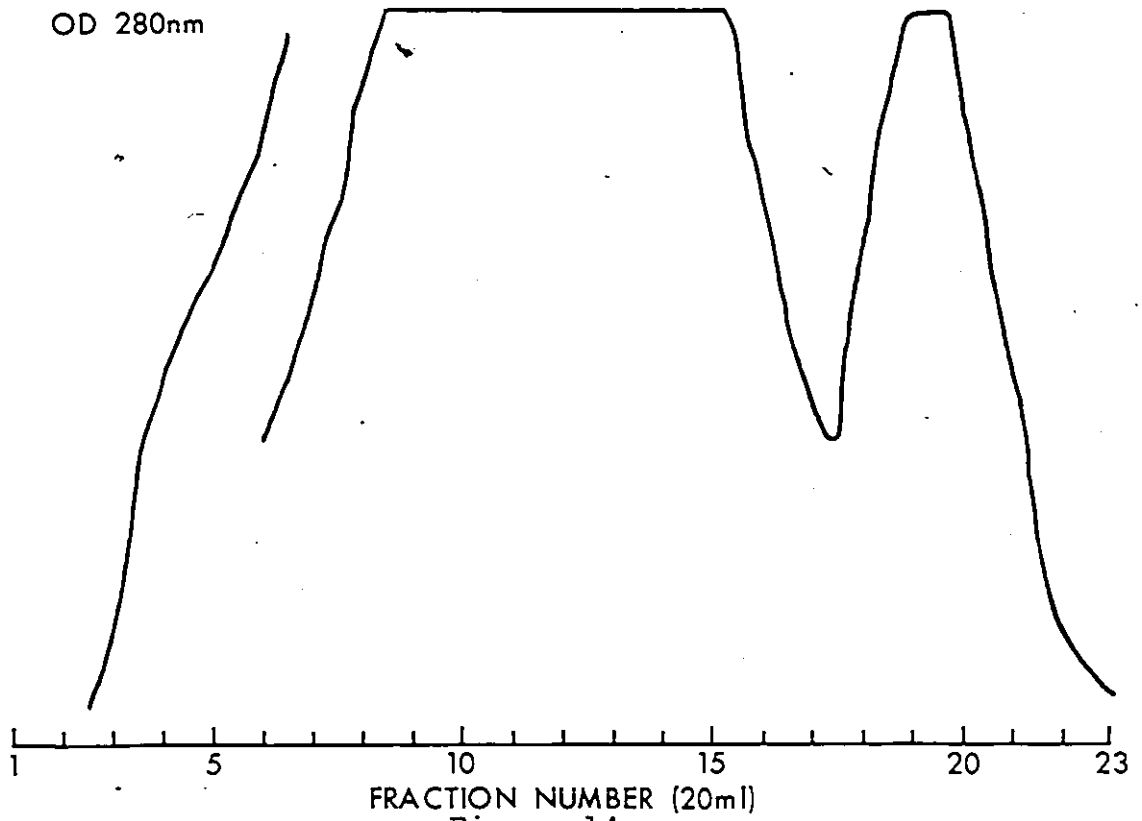


Figure 14

spherical particles with minimal contamination with short and long filaments.

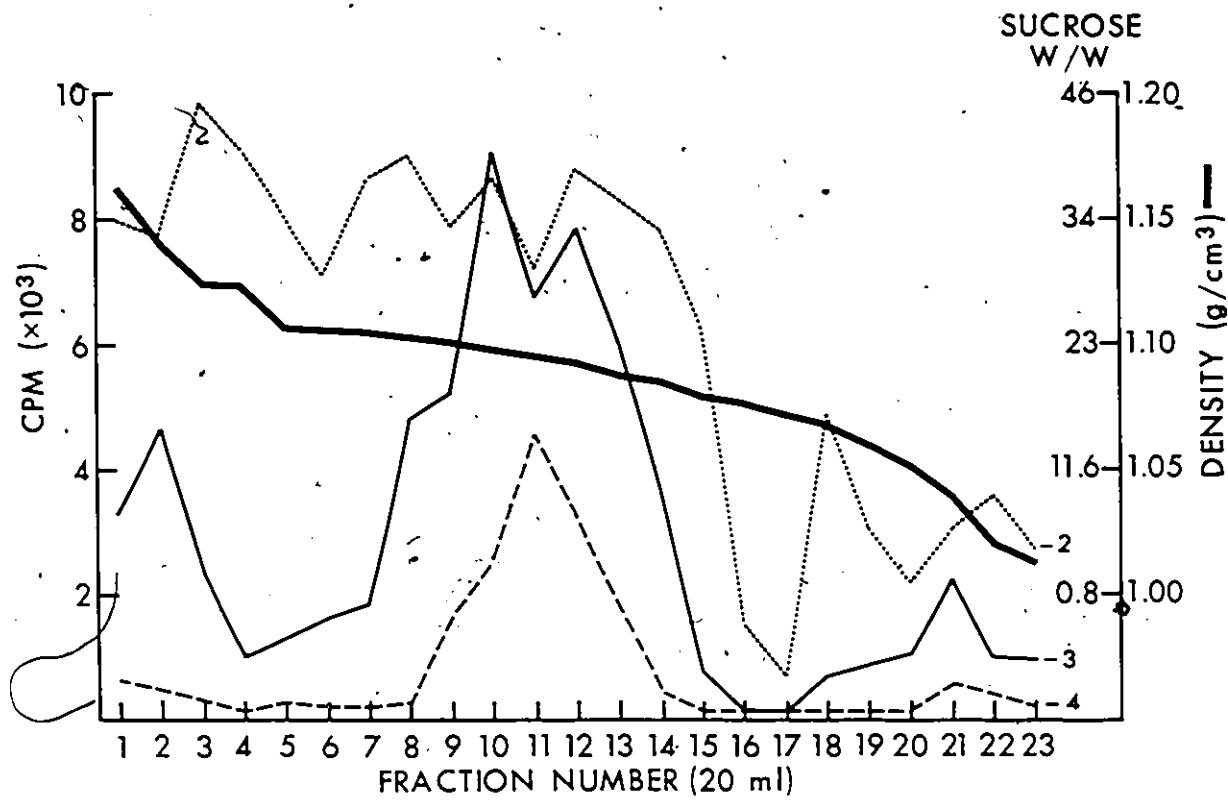
The peak fractions (19-21) from this second isopycnic banding were pooled, dialyzed against PBS in an Amicon 65 cell and concentrated to 45 ml as previously described. Twenty-five millilitres of CS2 were used for the rate separation centrifugation (CS2 R1). This procedure resulted in the coincident migration of RIA activity with a major optical density peak, well separated from the residual soluble serum contaminants which remained in the starting CS2 zone (figures 15, 16). Fractions 9-13 from the CS2 R1 banding were combined and rebanded in CsCl (CS2 R1 CS1). Optical density scanning at 280 nm and RIA screening at 10^{-4} dilution showed a single homogeneous peak at a density of 1.203 g/cm^3 (figures 17, 18). Fractions 10-13 were pooled, dialyzed extensively against PBS and concentrated to 50 ml in an Amicon 65 cell as previously described. The CS2 R1 CS1 sample was aliquoted in 1 ml volumes and stored at -70°C .

b) Small volume purification:--This procedure also involved 3 isopycnic bandings in discontinuous CsCl gradients and a rate separation centrifugation in a continuous sucrose gradient. The first isopycnic banding (CS1) had a peak CIEP reactivity for HB_sAg at a 1:128 titre and a buoyant density of 1.20 g/cm^3 (figure 19). Fractions 5-8 were pooled, adjusted to a density of 1.30 g/cm^3 as

Figures 15 and 16

15. The rate separation banding (CS2R1) in a continuous sucrose gradient.

16. Optical density determination at 280 nm of the CS2R1 banding. Peak RIA activity coincides with a peak composed of fractions 9-13.



• Figure 15

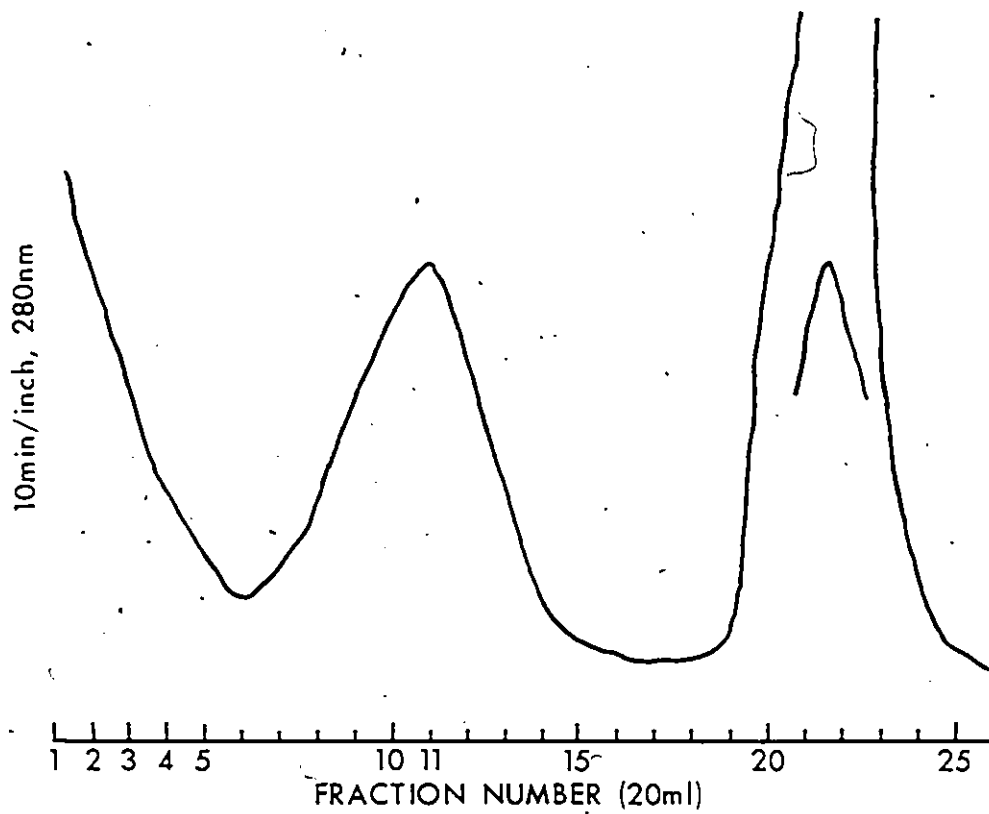


Figure 16

Figures 17 and 18

17. Third isopycnic banding in CsCl. Peak RIA activity coincides with a CsCl density of 1.203 g/cm^3 .
18. Optical density determination at 280 nm for the CS2R1 CS1 banding. The OD peak coincides with peak RIA activity.

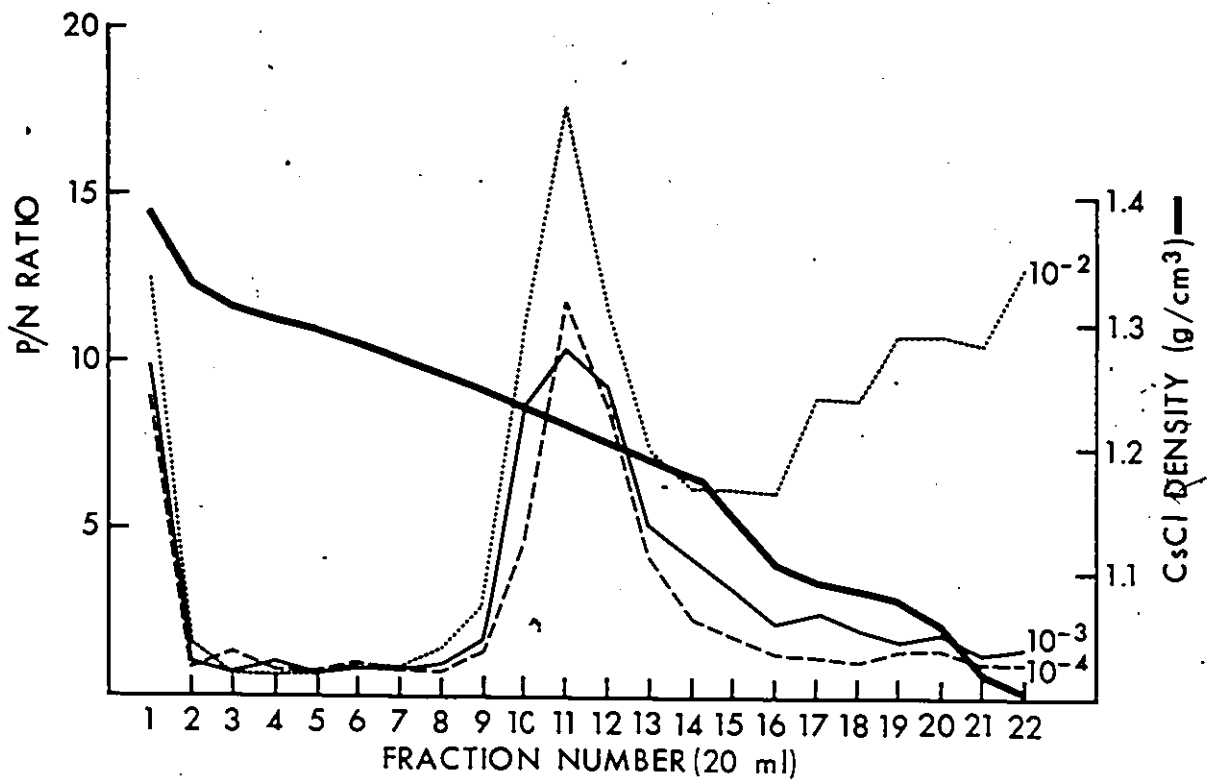


Figure 17

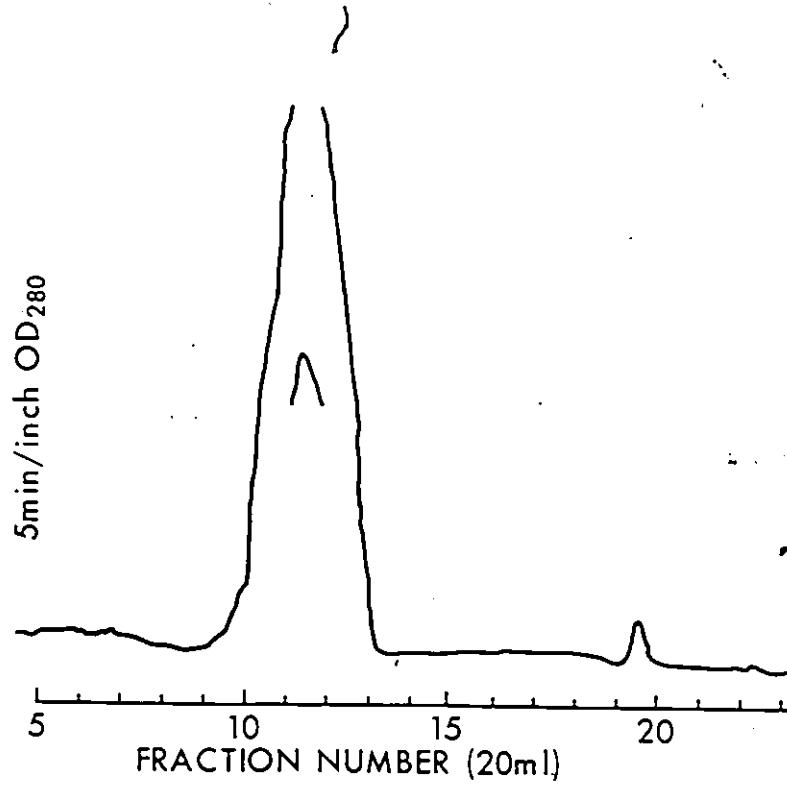


Figure 18

Figures 19 and 20

19. The first isopycnic banding in the small volume purification procedure. Peak CIEP activity is recovered at a density of 1.20 g/cm^3 in CsCl .
20. The CS2 banding in the small volume purification procedure. Peak CIEP activity coincides with the second UV peak obtained at 280 nm and is found at a density of 1.20 g/cm^3 in CsCl .

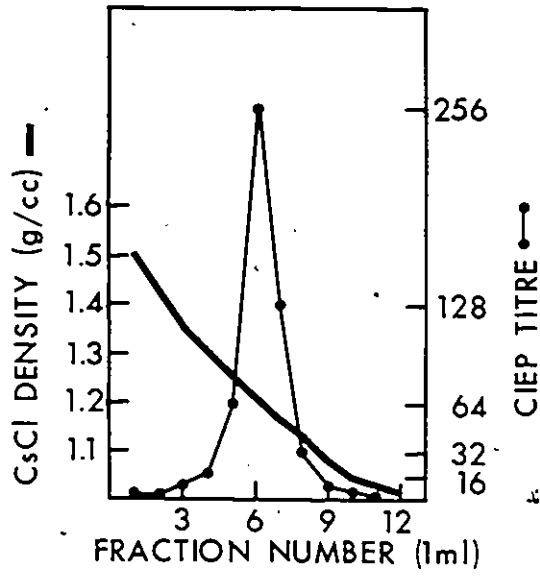


Figure 19

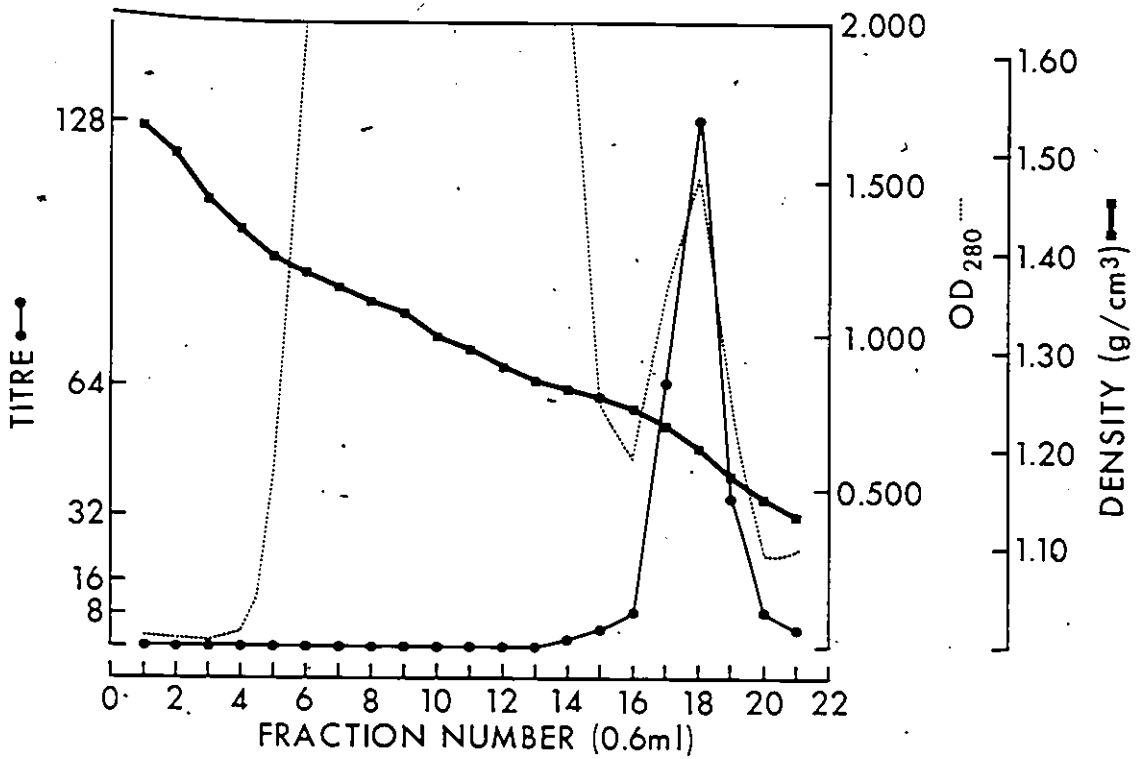


Figure 20

previously described and recentrifuged to float the 22 nm spherical particles away from the heavier serum protein contaminants. Peak CIEP activity was seen in the second 280 nm optical density zone and coincided with a 1.20 g/cm^3 buoyant density (figure 20). Fractions 17-19 of the CS2 banding were pooled, dialyzed against PBS and concentrated to 10 ml in an Amicon 65 cell. One millilitre aliquots per tube were used for the rate separation centrifugation (CS2 R1). Peak CIEP activity migrated with the first major 280 nm optical density zone, well away from the soluble serum contaminants which remained in the starting area at the top of the tube (figure 21). Fractions 13-17 were pooled and rebanded for a third time in CsCl (CS2 R1 CS1). Optimal CIEP activity was found in a single homogeneous peak of 280 nm absorption, with a buoyant density of 1.20 g/cm^3 (figure 22). Fractions 8-11 were pooled, dialyzed against PBS and dispensed in 1 ml aliquots for storage at -70°C .

c) Protein determination:--The protein concentrations of CS2 R1 CS1 preparations were determined by the Lowry technique. Protein concentrations of HB_sAg-purified material by the large volume procedure ranged between 110-275 $\mu\text{g/ml}$ whereas HB_sAg purified by the small volume method ranged between 100-245 $\mu\text{g/ml}$.

d) Electron microscopy:--EM examination of the CS2 R1 CS1 preparation showed a homogeneous population of

Figures 21 and 22

21. The rate separation centrifugation (CS2R1) in the small volume purification procedure.
22. The third isopycnic banding (CS2R1 CS1) in the small volume purification procedure. RIA activity is recovered in a single 280 nm peak.

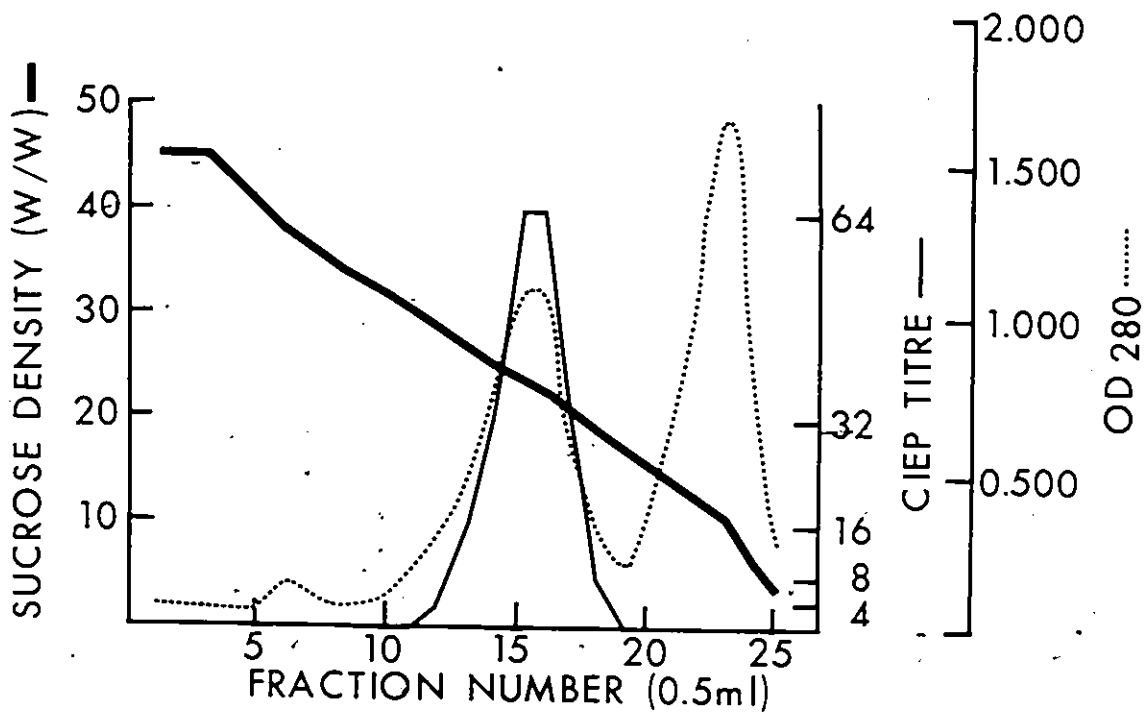


Figure 21

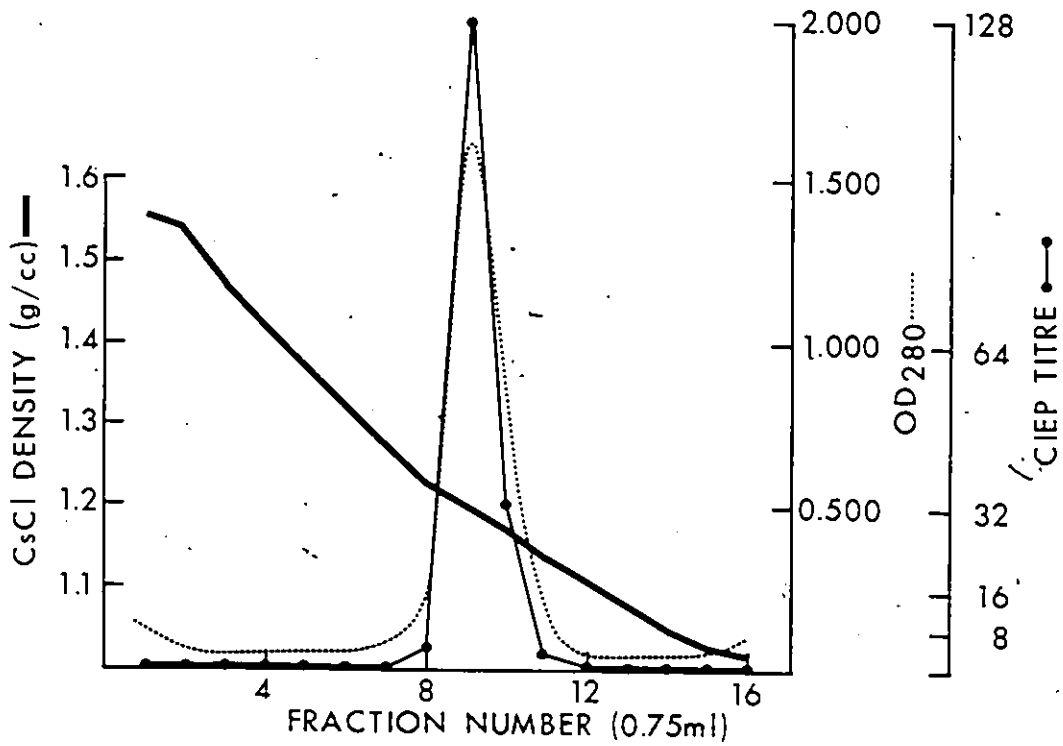


Figure 22

22 nm spherical particles. The number of particles per millilitre could not be estimated accurately as no standardized latex particle preparation was used for comparison (figs. 23a, b).

e) Subtype:--The subtypes of purified CS2 R1 CS1 preparations were identical to those of the starting sample in all cases. In addition, purification did not significantly enhance subtype determination for any HB_sAg-positive serum.

f) Assessment of CS2 R1 CS1 purity:--CIEP and AGD analyses of purified HB_sAg revealed no human serum contaminants in any of the 6 sera prepared by the large volume purification technique. Five of the 20 small-volume sera reacted with anti-NHS. These were reprocessed through the CS2 R1 and CS2 R1 CS1 steps and retested. These two rebandings were 100% successful in removing detectable contaminants.

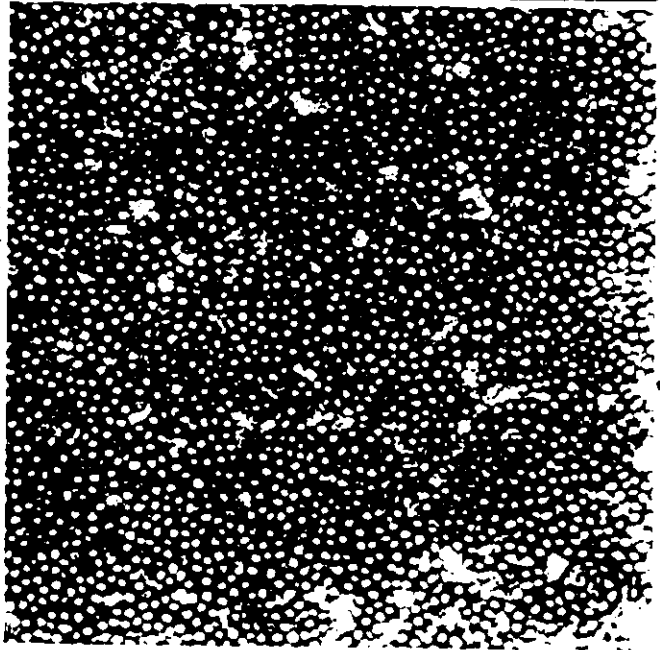
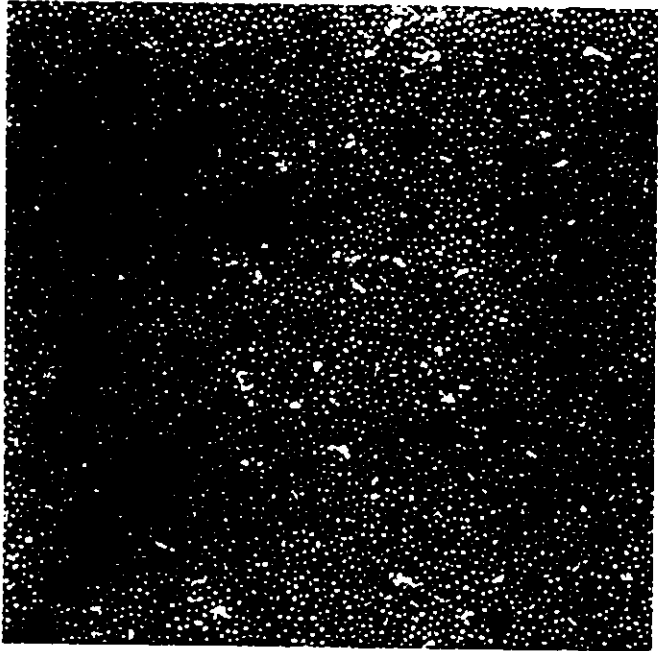
The antibody response of anti-HB_s negative guinea pigs to purified HB_sAg was assessed by AGD and CIEP. Detectable levels of anti-HB_s resulted 2-3 weeks after inoculation of 0.5 ml of HB_sAg (in complete Freund's adjuvant) into guinea pig foot pads. In addition, 3 of the 6 large volume preparations produced low but detectable titres of anti-NHS in these animals by AGD as did 8 of 20 small volume preparations. These titres ranged from undiluted (1:1) to 1:16. CS2 R1 CS1 preparations producing detectable anti-NHS titres in guinea pigs were stored at

Figures 23 a and b: A homogeneous population of 22 nm
spherical particles purified by the
small volume purification procedure:

a. Low magnification (140,000X)

b. Higher magnification (256,000X)

Bar represents 100 μ .



-70°C and not used for later CMI studies.

g) HB_s Ag purification following PEG precipitation:--

The CS1 and CS2 bandings of PEG-precipitated HB_s Ag were similar to the first 2 bandings described for the small volume purification procedure. CIEP activity was recovered in an opaque band following each isopycnic banding. Following the rate separation centrifugation, 3 major 280 nm optical density peaks were recoverable. The first peak, devoid of CIEP activity, contained PEG exclusively. The second peak was associated with CIEP activity and the third peak contained the soluble serum contaminants in the starting zone (figure 24). The baseline between these 3 peaks was markedly elevated when compared to the non-PEG precipitated HB_s Ag-preparations. This elevation was the result of PEG cross-contamination between the fractions and peaks. Repeated rate separation purifications of the CS2 R1 material reduced this baseline but PEG was always recoverable in all fractions. HB_s Ag titres by PEG-precipitation were often 2-4 fold higher than with the large and small volume purification procedures. Nevertheless, this technique was abandoned because of the problem of cross-contamination.

B. Hepatitis B_e antigen and antibody

1. Prevalence of HB_e Ag and anti-HB_e in four HB_s Ag-positive populations^e

As is shown in Table VII, a significant percentage of HB_s Ag-positive renal dialysis sera was also positive for

Figure 24: The rate separation banding from the
PEG-purification procedure

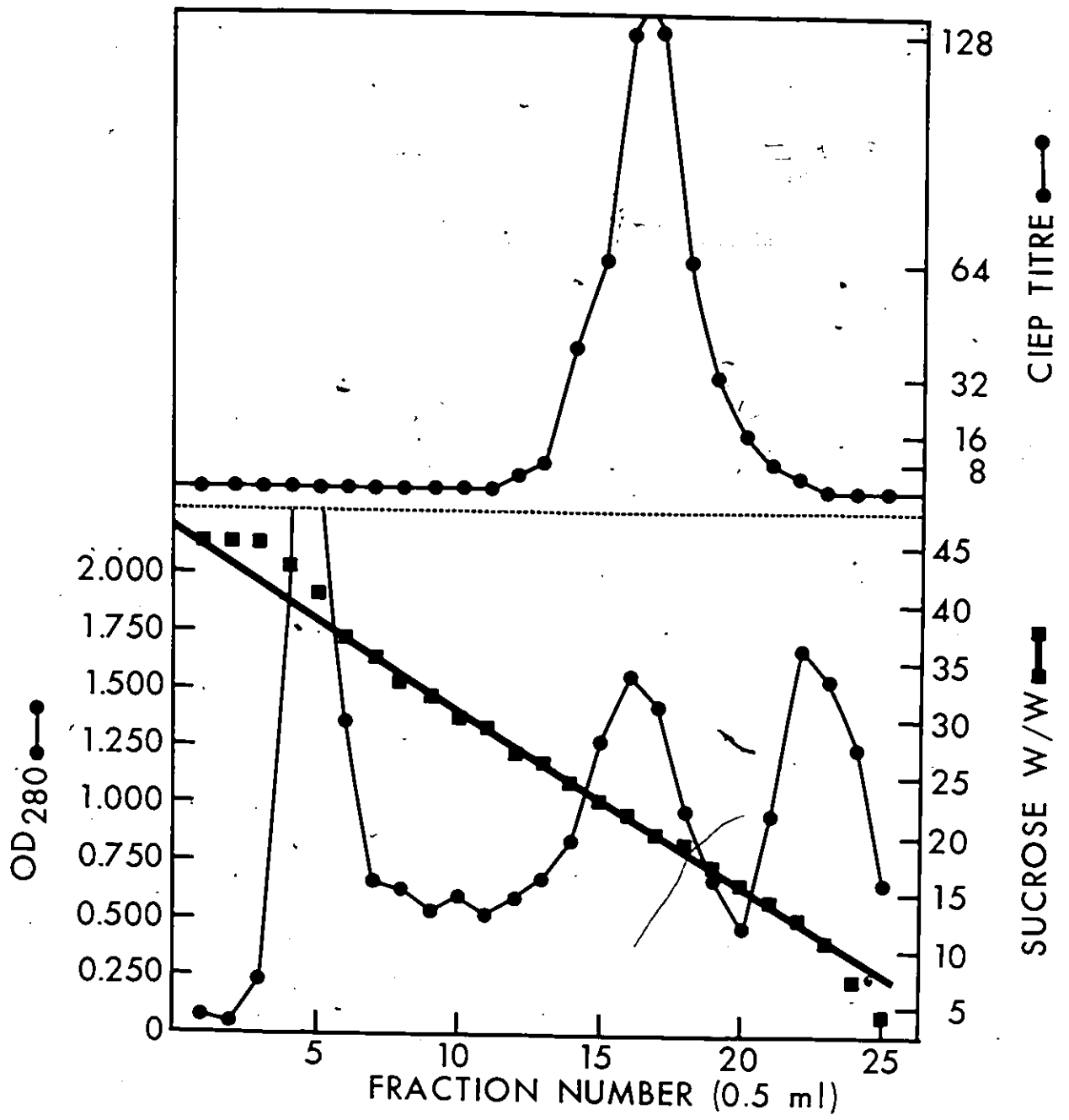


Figure 24

TABLE VII

PREVALENCE OF HB_eAg AND ANTI-HB_e IN FOURHB_sAg-POSITIVE POPULATIONS

Population	No. examined	No. HB _e Ag- positive	% HB _e Ag- positive	No. examined	No. anti-HB _e - positive	& anti-HB _e - positive
Down's syndrome (DS)	105	17	16.2	105	27	25.7
Other mentally retarded (OMR)	162	16	10.0	162	57	35.2
Blood donors (BD)	96	8	8.3	96	30	31.3
Renal dialysis _e (RD)	12	6	50.0	12	1	8.3

HB_eAg RD vs BD: χ^2 with continuity correction = 5.725, 0.05 > P > 0.01, d.f. = 1
 RD vs DS: χ^2 with continuity correction = 5.274, 0.05 > P > 0.01, d.f. = 1
 RD vs OMR: χ^2 with continuity correction = 5.070, 0.05 > P > 0.01, d.f. = 1
 All other comparisons: χ^2 with continuity correction P > 0.01 → P > 0.1.

Anti-HB_e RD vs BD, OMR and DS: χ^2 with continuity correction 0.05 > P > 0.01, d.f. = 1
 All other comparisons P > 0.1

e antigen by the AGD technique ($0.05 > p > 0.01$). Anti-HB_e on the other hand was detected in only 8.3% of HB_sAg-positive dialysis sera, significantly less than for the three other groups tested ($0.05 > p > 0.01$).

2. HB_eAg and anti-HB_e in two institutionalized populations.

a) Prevalence of HB_eAg and anti-HB_e in HB_sAg-positive sera:--As seen in Tables VIII and IX, there was no difference in the prevalence of e antigen and antibody between the DS and OMR populations at the two centres ($p > 0.1$).

b) Prevalence of HB_eAg and anti-HB_e in HB_sAg-positive patients as a function of age at admission:--Age at admission was not a contributory factor in the prevalence of either e antigen or antibody at the two institutions ($p > 0.1$). The findings are summarized in Tables X and XI.

c) Prevalence of HB_eAg and anti-HB_e in HB_sAg-positive patients as a function of length of residence:--

(1) HB_eAg. e antigen was more prevalent in the DS and OMR populations at the Rideau centre, and in the OMR population at the Huronia Centre when length of residence in these institutions was 10 years or less ($p < 0.05$). These results are shown in Table XII.

(2) Anti-HB_e. As is shown in Table XIII, anti-HB_e was not significantly affected by the length of residence in any of the populations at the two centres ($p > 0.1$).

TABLE VIII
 PREVALENCE OF HB_eAg IN HB_sAg-POSITIVE RESIDENTS OF TWO
 ONTARIO INSTITUTIONS FOR THE MENTALLY RETARDED

	No. of residents examined	Down's syndrome (DS)		Other mentally retarded (OMR)	
		No. examined	No. positive	No. examined	No. positive
			% positive		% positive
Rideau	146	54	10	92	8
Hurononia	121	51	7	70	8
			18.5		8.7
			13.7		11.4

DS vs OMR (Rideau): χ^2 with continuity correction = 2.486, $P > 0.1$, d.f. = 1
 DS vs OMR (Hurononia): χ^2 with continuity correction = 0.338, $P > 0.5$, d.f. = 1

TABLE IX
 PREVALENCE OF ANTI-HB_e IN HB_sAg-POSITIVE RESIDENTS OF TWO
 ONTARIO INSTITUTIONS FOR THE MENTALLY RETARDED

No. of residents examined	Down's syndrome (DS)			'Other mentally retarded' (OMR)		
	No. examined	No. positive	% positive	No. examined	No. positive	% positive
Rideau 146	54	12	22.2	92	35	38.0
Hurononia 121	51	15	29.4	70	24	34.3

DS vs OMR (Rideau): χ^2 with continuity correction = 2.962, $P > 0.1$, d.f. = 1
 DS vs OMR (Hurononia): χ^2 with continuity correction = 0.314, $P > 0.5$, d.f. = 1

TABLE X

PREVALENCE OF HB_sAg IN HB_sAg-POSITIVE RESIDENTS OF TWO ONTARIO INSTITUTIONS
FOR THE MENTALLY RETARDED BASED ON AGE AT ADMISSION

Age at admission (yrs)	Down's syndrome (DS)			Other mentally retarded (OMR)		
	No. examined	No. positive	% positive	No. examined	No. positive	% positive
Rideau						
0-10	43	7	16.3	74	4	5.4
11-20	11	3	27.3	16	4	25.0
>20	0	0	0	2	0	0
Total	54	10	18.5	92	8	8.7
Hurononia						
0-10	41	6	14.6	58	5	8.6
11-20	10	1	10.0	11	3	27.3
>20	0	0	0	1	0	0
Total	51	7	13.7	70	8	11.4

DS (Rideau): $\chi^2 = 0.948$, d.f. = 1, P > 0.5
OMR (Rideau): $\chi^2 = 3.342$, d.f. = 2, P > 0.1

DS (Hurononia): $\chi^2 = 0.158$, d.f. = 1, P > 0.5
OMR (Hurononia): $\chi^2 = 1.938$, d.f. = 2, P > 0.1

TABLE XI

PREVALENCE OF ANTI-HB_e IN HB_sAg-POSITIVE RESIDENTS OF TWO ONTARIO INSTITUTIONS
FOR THE MENTALLY RETARDED BASED ON AGE AT ADMISSION

Age at admission (yrs)	Down's syndrome (DS)			Other mentally retarded (OMR)		
	No. examined	No. positive	% positive	No. examined	No. positive	% positive
Rideau						
0-10	43	8	18.8	74	30	40.5
11-20	11	4	36.4	16	3	18.8
>20	0	0	0	2	2	100.0
Total	54	12	22.2	92	35	38.0
Hurononia						
0-10	41	12	29.2	58	20	34.5
11-20	10	3	30.0	11	3	27.3
>20	0	0	0	1	1	100.0
Total	51	15	29.4	70	24	34.3

DS (Rideau): $\chi^2 = 1.438$, d.f. = 1, $P > 0.1$
 OMR (Rideau): $\chi^2 = 4.142$, d.f. = 1, $P > 0.1$

DS (Hurononia): $\chi^2 = 0.273$, d.f. = 2, $P > 0.5$
 OMR (Hurononia): $\chi^2 = 2.791$, d.f. = 2, $P > 0.1$

TABLE XII

PREVALENCE OF HB_e Ag IN HB_s Ag-POSITIVE RESIDENTS OF TWO ONTARIO INSTITUTIONS
FOR THE MENTALLY RETARDED BASED ON LENGTH OF RESIDENCE

Length of residence (yrs)	Down's syndrome (DS)			Other mentally retarded (OMR)		
	No. examined	No. positive	% positive	No. examined	No. positive	% positive
Rideau						
0-10	7	5	71.4	18	6	33.3
11-20	33	5	15.2	35	2	5.7
>20	14	0	0	39	0	0
Total	54	10	18.5	92	8	8.7
Hurononia						
0-10	16	4	25.0	14	5	35.7
11-20	20	3	15.0	43	2	4.7
>20	15	0	0	13	1	7.7
Total	51	7	13.7	70	8	11.4

DS (Rideau): $\chi^2 = 4.324$, d.f. = 2, $0.05 > P > 0.01$ DS (Hurononia): $\chi^2 = 0.526$, d.f. = 2, $P > 0.1$
 OMR (Rideau): $\chi^2 = 6.261$, d.f. = 2, $0.05 > P > 0.01$ OMR (Hurononia): $\chi^2 = 6.710$, d.f. = 2, $0.05 > P > 0.01$

TABLE XIII

PREVALENCE OF ANTI-HB_e IN HB_s Ag-POSITIVE RESIDENTS OF TWO ONTARIO INSTITUTIONS
FOR THE MENTALLY RETARDED BASED ON LENGTH OF RESIDENCE

Length of residence (yrs)	Down's syndrome (DS)			Other mentally retarded (OMR)		
	No. examined	No. positive	% positive	No. examined	No. positive	% positive
Rideau						
0-10	7	1	14.3	18	5	27.8
11-20	33	7	21.3	35	9	25.4
>20	14	4	28.6	39	21	53.8
Total	54	12	22.2	92	35	38.0
Hurononia						
0-10	16	3	18.8	14	3	21.4
11-20	20	5	25.0	43	15	34.9
>20	15	7	46.7	13	6	46.2
Total	51	15	29.4	70	24	34.3

DS (Rideau): $\chi^2 = 0.616$, d.f. = 2, P > 0.5
OMR (Rideau): $\chi^2 = 4.087$, d.f. = 2, P > 0.1

DS (Hurononia): $\chi^2 = 1.804$, d.f. = 2, P > 0.1
OMR (Hurononia): $\chi^2 = 1.301$, d.f. = 2, P > 0.5

d) Prevalence of HB_eAg and anti-HB_e in HB_sAg-positive patients as a combined function of age at admission and length of residence:--

(1) HB_eAg. Most DS patients at both institutions were admitted between 0 and 10 years of age (95% and 84% at Rideau and Huronia respectively). Whereas 84% of the Rideau DS population were institutionalized for 10 or more years, only 53% at Huronia were residents for an equal period of time ($0.1 > p > 0.05$). A significant percentage of the Huronia population (31%) had been residents for less than 10 years ($0.05 > p > 0.01$). There were no such differences between the OMR populations at the two centres (Table XIIa).

Comparison of the DS and OMR patients admitted before the age of 10 and residing at the centre for more than 10 years showed a significantly higher percentage of e antigen in the DS population at the Rideau Centre only ($0.05 > p > 0.01$). When the length of residence was 10 years or less, the differences between the DS and OMR populations at the two centres were not significant ($p > 0.1$). These results are summarized in Tables XIIb and XIIc.

(2) Anti-HB_e. Age at admission and length of residence were found not to be significant factors in the prevalence of e antibody in either the DS or OMR populations at the two institutions (Tables XIIb and XIIc).

TABLE XIIa

HB Ag-POSITIVE POPULATIONS IN TWO INSTITUTIONS FOR THE RETARDED AS A FUNCTION
OF AGE AT ADMISSION (AA) AND LENGTH OF RESIDENCE (LR)

	Down's syndrome (DS)				Other mentally retarded (OMR)			
	No. examined	AA/LR 0-15/11-25 (%)	AA/LR 0-15/0-10 (%)	No. examined (%)	AA/LR 0-15/11-25 (%)	AA/LR 0-15/0-10 (%)	No. examined (%)	AA/LR 0-15/0-10 (%)
Rideau	54	45 (84)	6 (11)	92 (75)	69*	18 (20)		
Hurononia	51	27 (53)	16 (31)	70 (70)	49	11 (16)		

DS Rideau vs Huronia (AA/LR 0-15/11-25): χ^2 with continuity correction = 3.664, $0.05 > P > 0.01$, d.f.=1
(AA/LR 0-15/0-10): χ^2 with continuity correction = 5.973, $0.05 > P > 0.01$, d.f.=1

OMR Rideau vs Huronia (AA/LR 0-15/11-25): χ^2 with continuity correction = 0.125, $P > 0.5$, d.f. = 1
(AA/LR 0-15/0-10): χ^2 with continuity correction = 0.324, $P > 0.5$, d.f. = 1

TABLE XIIB

HB_e Ag AND ANTI-HB_e IN HB_s Ag-POSITIVE INSTITUTIONALIZED POPULATIONS--A FUNCTION OF AGE AT ADMISSION AND LENGTH OF RESIDENCE (AA 0-15; LR 11-25).

	Down's syndrome (DS)				Other mentally retarded (OMR)				
	No. examined	HB _e Ag (%)	anti-HB _e (%)	No. examined	HB _e Ag (%)	anti-HB _e (%)	No. examined	HB _e Ag (%)	anti-HB _e (%)
Rideau	45	5 (11)	11 (24)	69	1 (2)	28 (41)			
Hurononia	27	3 (11)	7 (26)	49	2 (4)	18 (37)			

HB_e Ag Rideau DS vs OMR: χ^2 with continuity correction = 5.268, $0.05 > p > 0.01$, d.f. = 1
 Hurononia DS vs OMR: χ^2 with continuity correction = 1.231, $p > 0.1$, d.f. = 1

Anti-HB_e $p > 0.5$ for both Rideau, Hurononia

TABLE XIIC

HB_e Ag AND ANTI-HB_e IN HB_s Ag-POSITIVE INSTITUTIONALIZED POPULATIONS--A FUNCTION OF AGE AT ADMISSION AND LENGTH OF RESIDENCE (AA 0-15; LR 0-10)

	Down's syndrome (DS)				Other mentally retarded (OMR)				
	No. examined	HB _e Ag (%)	anti-HB _e (%)	No. examined	HB _e Ag (%)	anti-HB _e (%)	No. examined	HB _e Ag (%)	anti-HB _e (%)
Rideau	6	4 (67)	1	18 (16)	6 (33)	5 (28)			
Huronis	-16	4 (25)	3 (18)	11	5 (45)	2 (18)			

HB_e Ag Rideau DS vs OMR: χ^2 with continuity correction = 1.167, P = 0.1, d.f. = 1
 Huronia DS vs OMR: χ^2 with continuity correction = 0.865, P = 0.1, d.f. = 1

Anti-HB_e P > 0.5

e) Prevalence of HB_eAg and anti-HB_e in matched HB_sAg-positive and HB_sAg/anti-HB_s negative pairs:--None of the 88 HB_sAg/anti-HB_s negative sera from DS and OMR residents at the Rideau Centre matched by age, sex and length of residence to a like number of HB_sAg-positive patients had detectable HB_eAg or anti-HB_e in their sera (p < 0.001). DS versus OMR comparisons for e antigen and antibody in the HB_sAg-positive group were not significant (Tables XIV and XV).

3. Characterization of HB_eAg and anti-HB_e

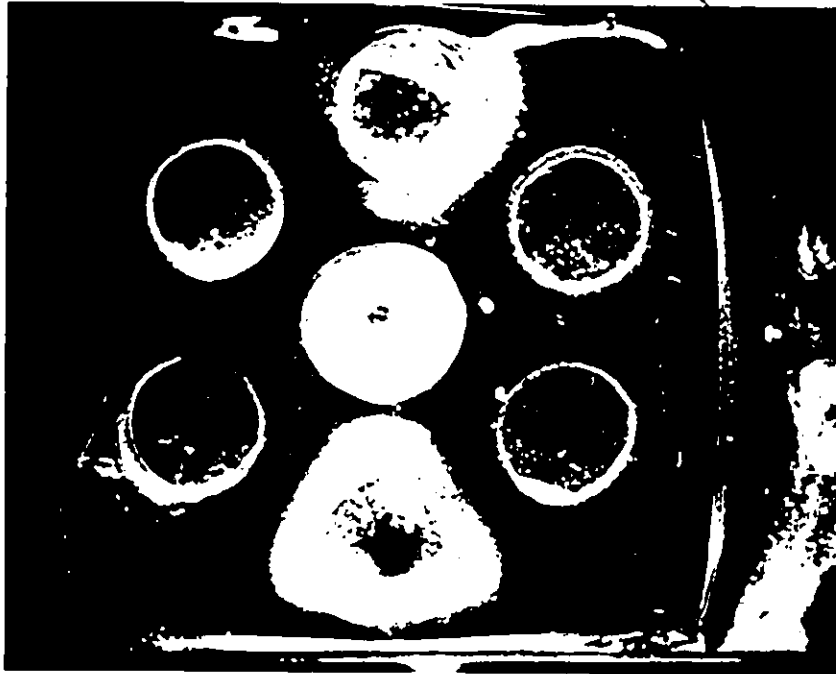
Two antigenic reactivities were found by AGD when a standard HB_eAg/e₁e₂ was tested against a known anti-HB_e/e₁e₂ (figure 25). One anti-HB_e gave 3 distinct precipitating lines with e₁e₂ antigen-positive sera, and was designated the Huronia antiserum (figure 26). In no instances were three lines seen when the antigen was an e₁ subtype. HB_eAg and anti-HB_e were not detected in any HB_sAg/anti-HB_s negative sera screened.

a) Heat inactivation of HB_eAg-positive sera:--
HB_eAg/e₁ and HB_eAg/e₁e₂ containing sera were heat inactivated at 56°C for 30 minutes and tested against anti-HB_e/e₁e₂ by immunodiffusion. The e₁ line was detectable in 90% of the sera, but the e₁ titre fell by as much as 50%. The e₂ line was not detectable in any of the inactivated HB_eAg/e₁e₂ sera. Sera giving 3 lines with the Huronia antiserum were also heat inactivated and only the e₁ line persisted. Because of the similarity of the third line with e₂ appearance and

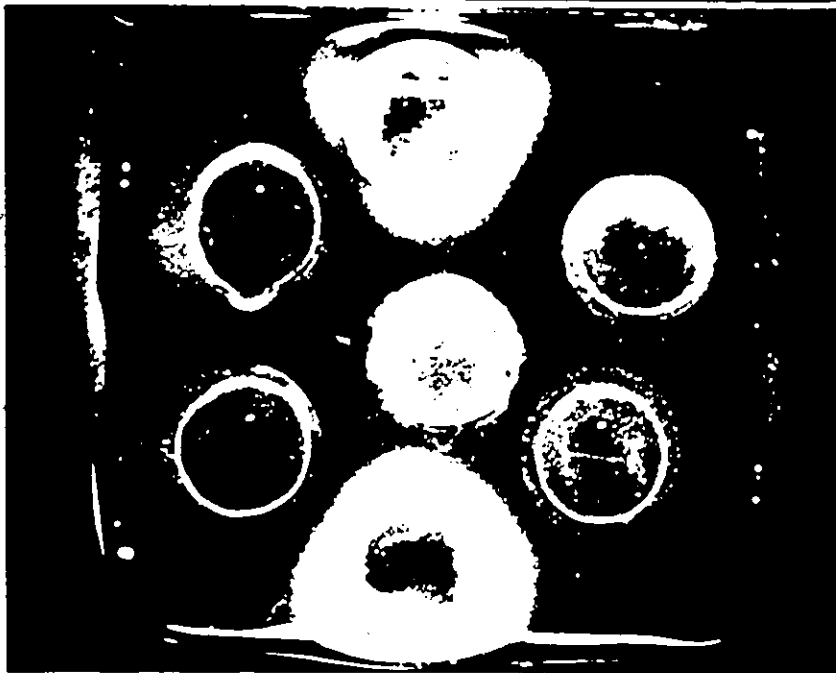
Figures 25 and 26

25. $HB_c Ag/e_1 e_2$

26. Three lines are produced when known $HB_e Ag/e_1 e_2$ and anti- $HB_e/e_1 e_2$ positive sera are tested against the Huronia antiserum. Because of the similarities between e_2 and the third line, it is designated e_{2_a} rather than e_3 .



25



26

TABLE XIV

HB_eAg IN HB_sAg-POSITIVE AND HB_sAg/ANTI-HB_s-NEGATIVE POPULATIONS AT THE RIDEAU REGIONAL CENTRE MATCHED BY MENTAL STATUS, AGE AT ADMISSION AND LENGTH OF RESIDENCE

No. of patients matched	Down's syndrome (DS)		Other mentally retarded (OMR)	
	No. examined	No. positive %	No. examined	No. positive %
HB _s Ag- positive 88	19	4 21.1	69	6 8.7
HB _s Ag/anti- HB _s -nega- tive 88	19	0 0	69	0 0

DS vs OMR: χ^2 with continuity correction = 1.669, $P > 0.1$, d.f. = 1

TABLE XV

ANTI-HB_e IN HB_SAg-POSITIVE AND HB_SAg/ANTI-HB_S-NEGATIVE POPULATIONS AT
 THE RIDEAU REGIONAL CENTRE MATCHED BY MENTAL STATUS, AGE AT
 ADMISSION AND LENGTH OF RESIDENCE

No. of patients matched	Down's syndrome (DS)			Other mentally retarded (OMR)			
	No. examined	No. positive	% positive	No. examined	No. positive	% positive	
HB _S Ag-positive	88	19	4	21.1	69	30	43.5
HB _S Ag/anti-HB _S -negative	88	19	0	0	69	0	0

DS vs OMR: χ^2 with continuity correction = 2.541, $p > 0.1$, d.f. = 1

disappearance, it was designated e_{2a} .

b) HB_eAg and anti-HB_e subtypes in HB_sAg-positive sera:--

(1) HB_eAg. All Rideau DS and OMR HB_eAg-positive sera were e_1 subtype as were all Huronia DS sera positive for e antigen. Of the eight HB_eAg-positive OMR sera from the Huronia centre, 7 were e_1 and 1 was e_1e_2 (Table XVI).

(2) Anti-HB_e. The majority of anti-HB_e positive sera were anti- e_1 subtype, followed in descending frequency by anti- e_1e_2 and finally anti- e_2 . One serum from Huronia gave 3 precipitating lines as already described and was designated anti- $e_1e_2e_{2a}$. Results are summarized in Table XVII.

4. Relationship of HB_eAg and anti-HB_e to HB_sAg subtypes

Blood donor sera positive for HB_sAg were subtyped and tested for e antigen and antibody. HB_eAg was detected in 15.6% of HB_sAg/ay sera compared to only 3.9% for the ad subtype ($0.05 > p > 0.01$). In renal dialysis patients, 50% of the HB_sAg/ad sera were also positive for the e antigen. All HB_sAg/ad sera that were e antigen positive were of the e_1 subtype whereas two of the seven ay subtypes were HB_eAg/ e_1e_2 (Table XVIII).

Anti-HB_e was most prevalent in blood donor HB_sAg/ad sera ($0.1 > p > 0.05$) and least detected in dialysis patients of the same subtype (Table XIX).

TABLE XVI

HB_e Ag SUBTYPES IN HB_s Ag-POSITIVE RESIDENTS OF TWO INSTITUTIONS
FOR THE MENTALLY RETARDED IN ONTARIO

	Down's syndrome (DS)				Other mentally retarded (OMR)			
	No. examined	e ₁	e ₂	e ₁ e ₂ Other	No. examined	e ₁	e ₂	e ₁ e ₂ Other
Rideau	10	10	-	-	8	8	-	-
Hurononia	7	7	-	-	8	7	-	1

TABLE XVII

ANTI-HB_e SUBTYPES IN HB_s Ag-POSITIVE RESIDENTS OF TWO ONTARIO INSTITUTIONS FOR THE MENTALLY RETARDED

	Down's syndrome (DS)				Other mentally retarded (OMR)			
	No. examined	e ₁	e ₂	e ₁ e ₂ Other	No. examined	e ₁	e ₂	e ₁ e ₂ Other
Rideau	12	8	2	2 -	35	21	3	10 1
Huron	15	12	-	3 -	24	16	1	7 -

TABLE XVIII

RELATIONSHIP OF HB_sAg SUBTYPES ad AND ay TO HB_eAg IN
BLOOD DONOR AND RENAL DIALYSIS POPULATIONS

Population	<u>ad</u>			<u>ay</u>		
	No. examined	No. positive	% positive	No. examined	No. positive	% positive
Blood donors (BD)	51	2 ¹	3.9	45	7 ²	15.6
Renal dialysis patients (RD)	12	6 ¹	50.0	0	-	-

¹ All HB_eAg subtype e₁

² 5-e₁, 2-e₁e₂

BD: ad vs ay with continuity correction = 4.159, 0.05 > P > 0.01, d.f. = 1

TABLE XIX

RELATIONSHIP OF HB_sAg SUBTYPES ad AND ay TO ANTI-HB_e IN
BLOOD DONOR AND RENAL DIALYSIS POPULATIONS

Population	ad			ay		
	No. examined	No. positive	% positive	No. examined	No. positive	% positive
Blood donors (BD)	51	21 ¹	41.2	45	9 ²	20.0
Renal dialysis patients (RD)	12	1 ³	8.3	0	-	-

1 14-e₁; 6-e₁e₂; 1-e₂

2 6-e₁; 3-e₁e₂

3 1-e₁

BD: ad vs ay with continuity correction = 3.782, 0.1 > P > 0.05, d.f. = 1

C. DNA polymerase

1. Assay (DPA)

The results of the DPA on sera obtained from 75 asymptomatic HB_sAg positive carriers (25 HB_eAg positive, 25 anti-HB_e positive and 25 negative for both HB_eAg and anti-HB_e by AGD) are summarized in table XX. The counts per minute (cpm) of 7 unconcentrated normal sera, negative for both HB_sAg and anti-HB_s and treated in an identical manner as the 75 HB_sAg positive sera, fell within the range of 19 and 70 cpm (mean ± standard deviation, 40 ± 9.2). Sera were considered to be positive for DNA polymerase activity when the cpm exceeded the mean of the 7 normal controls plus 5 standard deviations of the mean.

DNA polymerase activity was detected in 11 (44%) of the unconcentrated HB_eAg positive sera and in 20 (80%) of the 25 sera samples after 5 times concentration. In sharp contrast, none of the 25 anti-HB_e-positive sera had any detectable DNA polymerase activity, either in unconcentrated or concentrated form. The third group of HB_sAg-positive sera tested were negative for both HB_eAg and anti-HB_e by AGD. DNA polymerase activity was detected in 4 (16%) of the unconcentrated sera and in 9 (36%) sera following 5 times concentration. A total of 15 (20%) unconcentrated HB_sAg-positive sera were found to have DNA polymerase activity, increasing to 29 (39%) after concentration of the serum samples.

TABLE XX
 RELATIONSHIP OF HB_S Ag-ASSOCIATED DNA POLYMERASE ACTIVITY
 AND THE e ANTIGEN/ANTI-HB_e SYSTEM IN SERUM SAMPLES
 OF ASYMPTOMATIC HB_S Ag CARRIERS

HB _e Ag or anti-HB _e	DNA polymerase assay				
	No.	[1x Serum]		[5x Serum]	
		Positive (%) ¹	Negative (%)	Positive (%) ¹	Negative (%)
HB _e Ag	25	11 (44)	14 (56)	20 (80)	5 (20)
anti-HB _e	25	0	25 (100)	0	25 (100)
²	25	4 (16)	21 (84)	9 (36)	16 (64)
Total	75	15 (20)	60 (80)	29 (39)	46 (61)

¹Uptake of [³H]TTP greater than 76 cpm (mean count of 7 normal sera + 5 S.D. - 40 ± 9.2).

²HB_eAg and anti-HB_e negative by immunodiffusion.

The 20 HB_eAg sera positive for DNA polymerase activity were further tested for HB_sAg subtype and titre. Twelve of the sera were HB_sAg/ad while the remaining 8 were HB_sAg/ay. Titres for the sera ranged between 1:16 and 1:256 by CIEP. Mean DPA cpm for the 12 unconcentrated HB_sAg/ad sera were 112.5; the mean cpm for the 8 ay sera were 153.4. There was a significant increase in ³H-TTP incorporation following 5 times concentration of the individual serum samples; mean cpm increased to 730.2 for the 12 HB_sAg/ad sera and 1803.8 for the 8 ay sera (p < 0.001). The difference in ³H-TTP incorporation between the 2 subtypes was also significant (p < 0.001). In addition, these 20 sera were examined by EM for the presence of Dane particles, and were scored as 1+ to 4+, depending on the number of Dane particles per field (see Table XXI). Both empty and full particles were seen in all 20 sera following negative staining with 2% PTA (figure 27). Four of the 8 HB_sAg/ay sera contained more than 75 Dane particles per field (i.e., 4+) but only 1 of 12 HB_sAg/ad sera contained a like number of Dane particles. Numerous long filaments and 22 nm spherical particles were also seen in all samples. These results are summarized in Table XXI.

Twenty anti-HB_e positive sera, matched for HB_sAg subtype but negative for DNA polymerase activity in unconcentrated form were also retested for ³H-TTP uptake following 5 times concentration. None of the 20 sera were positive for DNA polymerase activity following concentration, with

TABLE XXI

DNA POLYMERASE ACTIVITY AND DANE PARTICLE COUNTS IN 20

SERUM SAMPLES OF ASYMPTOMATIC HB_e Ag CARRIERS

HB _s Ag Subtype	Titre (CIEP)	DNA polymerase activity [³ H]TTP uptake - cpm		Dane particles ¹ (number/ EM field)
		[1x]	[5x]	
ad	16	53	447	+
ad	64	159	656	++
ad	32	122	844	+++
ad	64	193	1114	+++
ad	32	129	760	++
ad	16	59	545	+
ad	16	62	478	++
ad	128	259	2156	++++
ad	32	57	432	++
ad	16	65	517	++
ad	32	66	320	+
ad	64	127	493	+
ay	256	469	4620	++++
ay	64	122	1460	++++
ay	128	236	2775	++++
ay	64	87	930	+++
ay	16	50	725	++
ay	32	69	660	++
ay	16	61	715	++
ay	64	133	2545	++++

Mean ± SD

ad

ay

[1x]

112.5 ± 65.1

153.4 ± 141.4

[2x]

730.2 ± 498.7

1803.8 ± 1413.1

¹Number of Dane particles/EM field.

+	<10
++	10 - 25
+++	26 - 75
++++	>75

individual serum sample cpm occasionally decreasing to the negative value range (serum sample cpm--mean of the negative controls). Two HB_sAg/ay were positive for Dane particles, but contained less than 10 particles per field (i.e., 1+); moreover particles in these 2 samples were empty. No Dane particles were seen in any of the 12 anti-HB_e-positive HB_sAg/ad sera (Table XXII).

2. Specificity (DPSA)

Sera positive for DNA polymerase activity were tested for specificity as described in Materials and Methods. The assay was considered specific when guinea pig anti-HB_s reduced the supernatant counts per minute by more than 50%. Treatment with normal guinea pig serum and PBS, on the other hand, produced no significant decrease in counts. High cpm following treatment with guinea pig anti-HB_s indicated non-specific DNA polymerase activity, usually caused by bacterial contamination of the serum tested. This occurred only when a serum sample had not been filtered prior to testing for DNA polymerase activity (Table XXIII).

All 20 sera tested for DNA polymerase specificity were found to contain specific hepatitis B DNA polymerase activity with a reduction in cpm of more than 50% following treatment with guinea pig anti-HB_s (range 66 - 93%). There was no significant decrease in cpm after treatment with either normal guinea pig serum or PBS (Table XXIV).

TABLE XXII

DNA POLYMERASE ACTIVITY AND DANE PARTICLE COUNTS IN 20
SERUM SAMPLES OF ASYMPTOMATIC ANTI-HB_e CARRIERS

HB _s Ag Subtype	Titre (CIEP)	DNA polymerase activity [³ H]TTP uptake - cpm		Dane particles ¹ (number/ EM field)
		[1x]	[5x]	
ad	32	24	16	-
ad	16	57	12	-
ad	64	29	9	-
ad	32	10	10	-
ad	32	34	4	-
ad	16	34	7	-
ad	16	17	5	-
ad	64	47	-3	-
ad	128	41	-10	-
ad	32	29	-12	-
ad	64	16	-11	-
ad	16	22	-1	-
ay	64	51	9	+
ay	16	27	6	-
ay	32	30	5	-
ay	64	25	4	+
ay	128	42	-30	-
ay	32	15	-6	-
ay	16	10	-12	-
ay	16	12	-27	-

Mean (\bar{X})	[1x]	[5x]
ad	30 ± 13.8	2.2 ± 9.5
ay	26.5 ± 14.3	-6.3 ± 15.3

¹Number of Dane particles/EM field

+	<10
++	10 - 25
+++	26 - 75
++++	>75

TABLE XXIII

INTERPRETATION OF THE DNA POLYMERASE

SPECIFICITY ASSAY

Sample supernatant	Expected Values CPM in supernatants after 180 minutes incubation		
	A ¹	Tubes B	C
1) HBV ² ; no bacteria (filtered)	Low ³	High	High
2) HBV-; no bacteria	Low	Low	Low
3) HBV+; bacteria+(not filtered)	High	High	High
4) HBV-; bacteria+	High	High	High

¹ A--treated with guinea pig anti-HB_s serum.

B--treated with normal guinea pig serum.

C--treated with phosphate buffered saline (PBS).

² Hepatitis B virus--HB_s Ag⁺; HB_e Ag⁺; Dane particles⁺

³ Low count means a reduction by more than 50%.

TABLE XXIV

DNA POLYMERASE SPECIFICITY ASSAY FOR 20

HB_e Ag POSITIVE SERA

Sample	CPM [5x]	³ H-TTP counts per minute in supernatants after 180 minutes incubation		
		A ¹	Tubes B	C
ad	447	37 (92) ²	417	421
ad	656	48 (93)	573	558
ad	844	131 (84)	849	799
ad	1114	262 (76)	1010	1055
ad	760	135 (82)	750	775
ad	545	105 (81)	560	522
ad	478	113 (76)	418	429
ad	2156	675 (69)	1830	1970
ad	432	112 (74)	390	445
ad	517	76 (85)	502	530
ad	320	19 (94)	300	330
ad	493	60 (88)	460	490
ay	4620	816 (82)	3700	4065
ay	1460	502 (66)	1030	1420
ay	2775	595 (78)	2035	2620
ay	930	214 (77)	970	830
ay	725	110 (85)	720	815
ay	660	71 (89)	515	730
ay	715	190 (73)	630	610
ay	2545	405 (84)	2620	2815

¹A--treated with guinea pig anti-HB_s.

B--treated with normal guinea pig serum.

C--treated with PBS.

²% reduction in cpm.

3. HB_eAg and DNA polymerase in the course of an acute hepatitis B illness

Two individuals who contracted acute hepatitis B were followed during the course of their illnesses and for several months after resolution. The first individual (JD) was a nurse in a renal dialysis unit where staff were screened routinely on a monthly basis for HB_sAg by RIA. Serial observations of this patient's illness are shown in figure 28. HB_sAg was first detected on January 11 after suspected exposure in early December; it persisted for about four and a half months. The antigen was subtyped as HB_sAg/ad, the predominant subtype of the chronically infected dialysis patients. HB_eAg/e₁ and DNA polymerase activity were detected on February 7, with HB_eAg/e₂ appearing one week later at the peak of DNA polymerase activity. These three parameters were transient, disappearing within two and a half weeks after initial detection. Transaminase and bilirubin values^d were elevated for approximately six and four weeks respectively whereas alkaline phosphatase remained within normal limits throughout the course of the disease. Anti-HB_c first became detectable concomitantly with HB_eAg and DNA polymerase activity and persisted for the remaining period of observation. Anti-HB_e was detected approximately two and a half months after the disappearance of HB_eAg but was not detectable by AGD four months later. Anti-HB_s was detectable by RIA five months after resolution of antigenemia and it too persisted for the remaining period of observation.

Figures 28 and 29

28. Serial observations of HB_sAg, HB_eAg and DNA polymerase in a patient (J.D.) with acute HB_sAg/ad hepatitis.
29. Serial observations of HB_sAg, HB_eAg and DNA polymerase in a patient (D.K.) with acute HB_sAg/ay hepatitis.

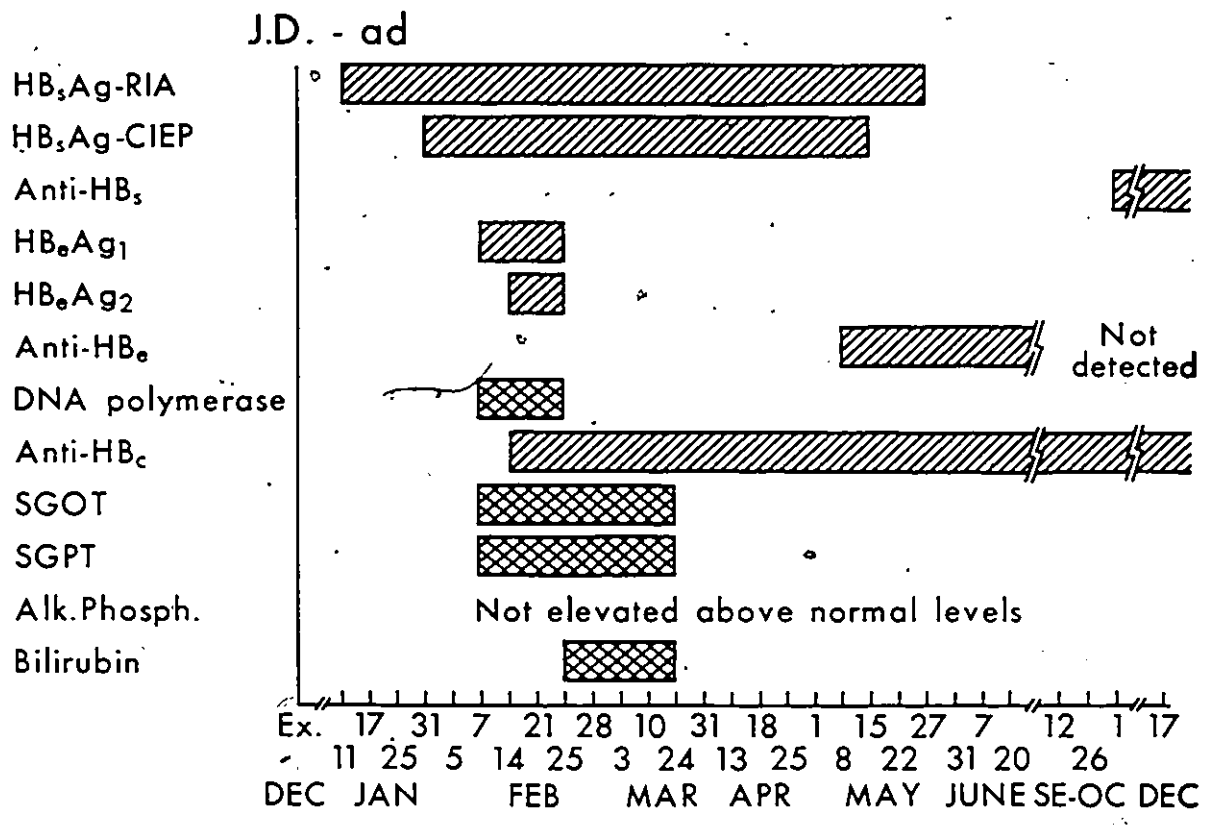


Figure 28

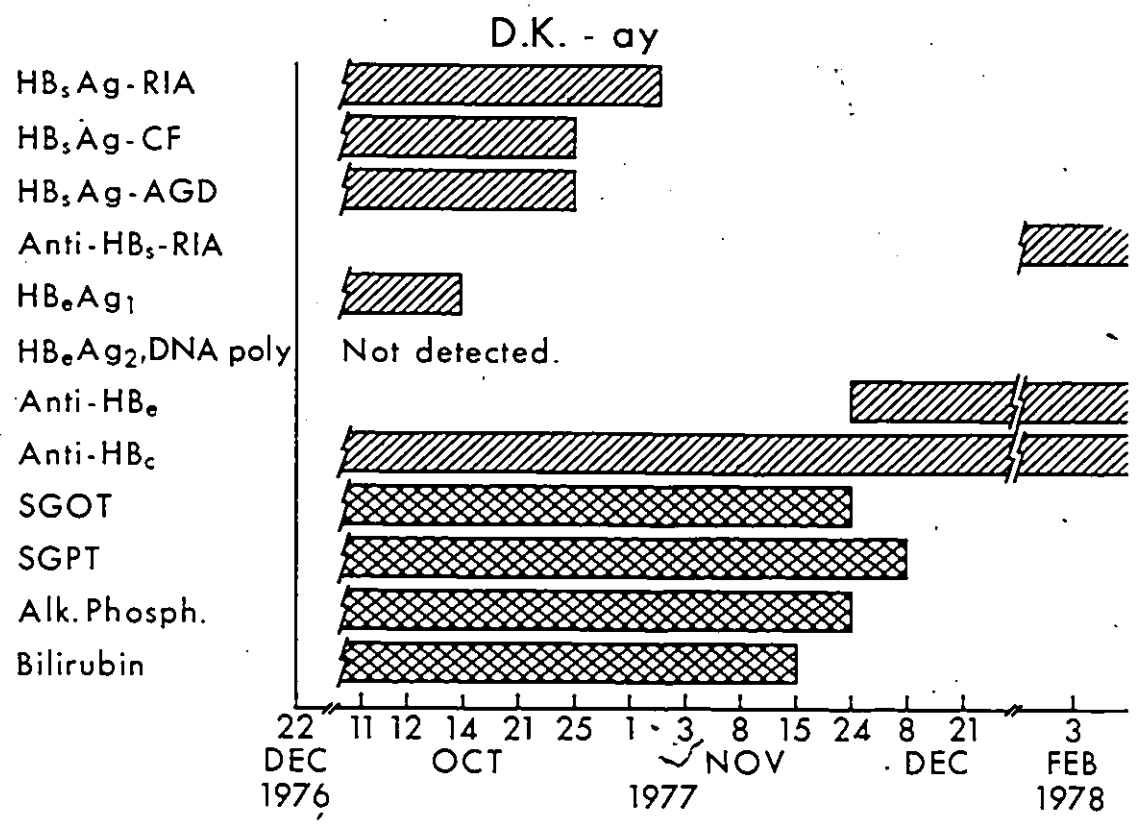


Figure 29

The second individual (DK) was a researcher who worked extensively with hepatitis B. Unfortunately, serum samples were only collected after the patient became icteric and no estimate of the time of exposure was available. The antigen subtype was HB_sAg/ay and the course of this patient's illness is diagrammed in figure 29. HB_sAg persisted for an additional three and a half weeks after the onset of jaundice. HB_eAg/e₁ was detectable for only three days with no HB_eAg/e₂ and DNA polymerase activity detected. Transaminase, alkaline phosphatase and bilirubin values were elevated for five to eight weeks. Anti-HB_c was detected in the first sample tested and persisted for the entire period of observation. Anti-HB_e was detected six weeks after the disappearance of HB_eAg but anti-HB_s only appeared three months after clearance of HB_s antigenemia. Both persisted for the remainder of the observation period.

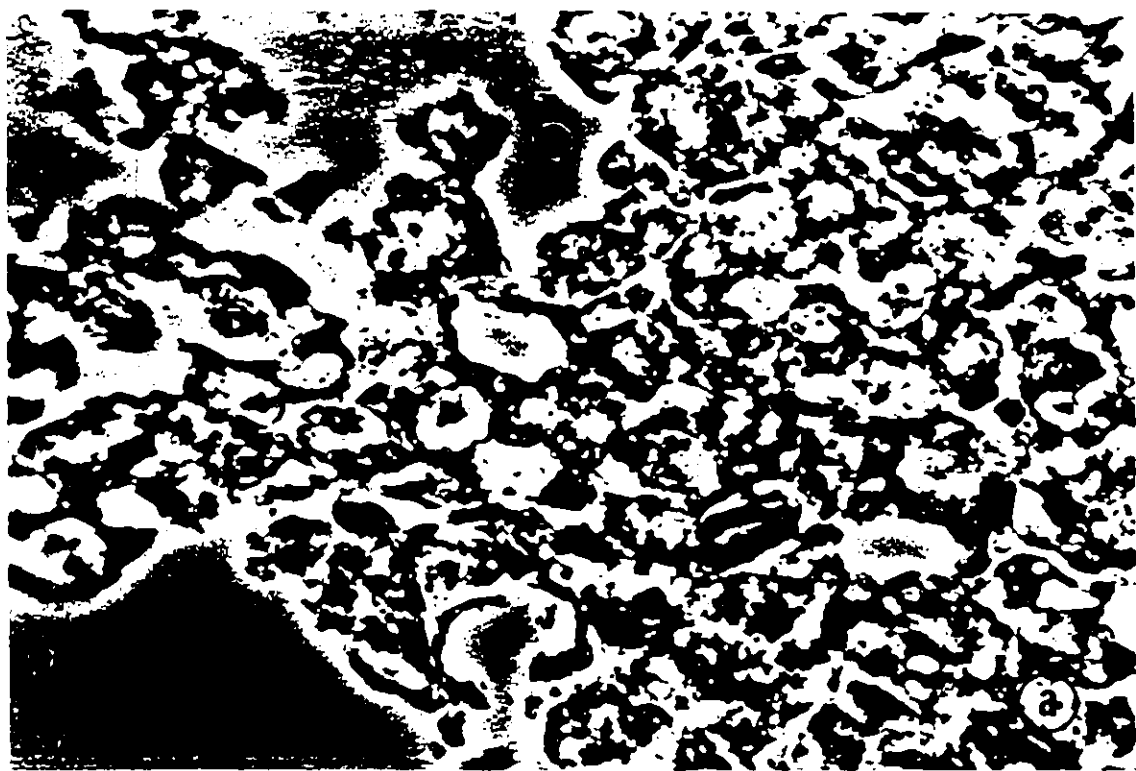
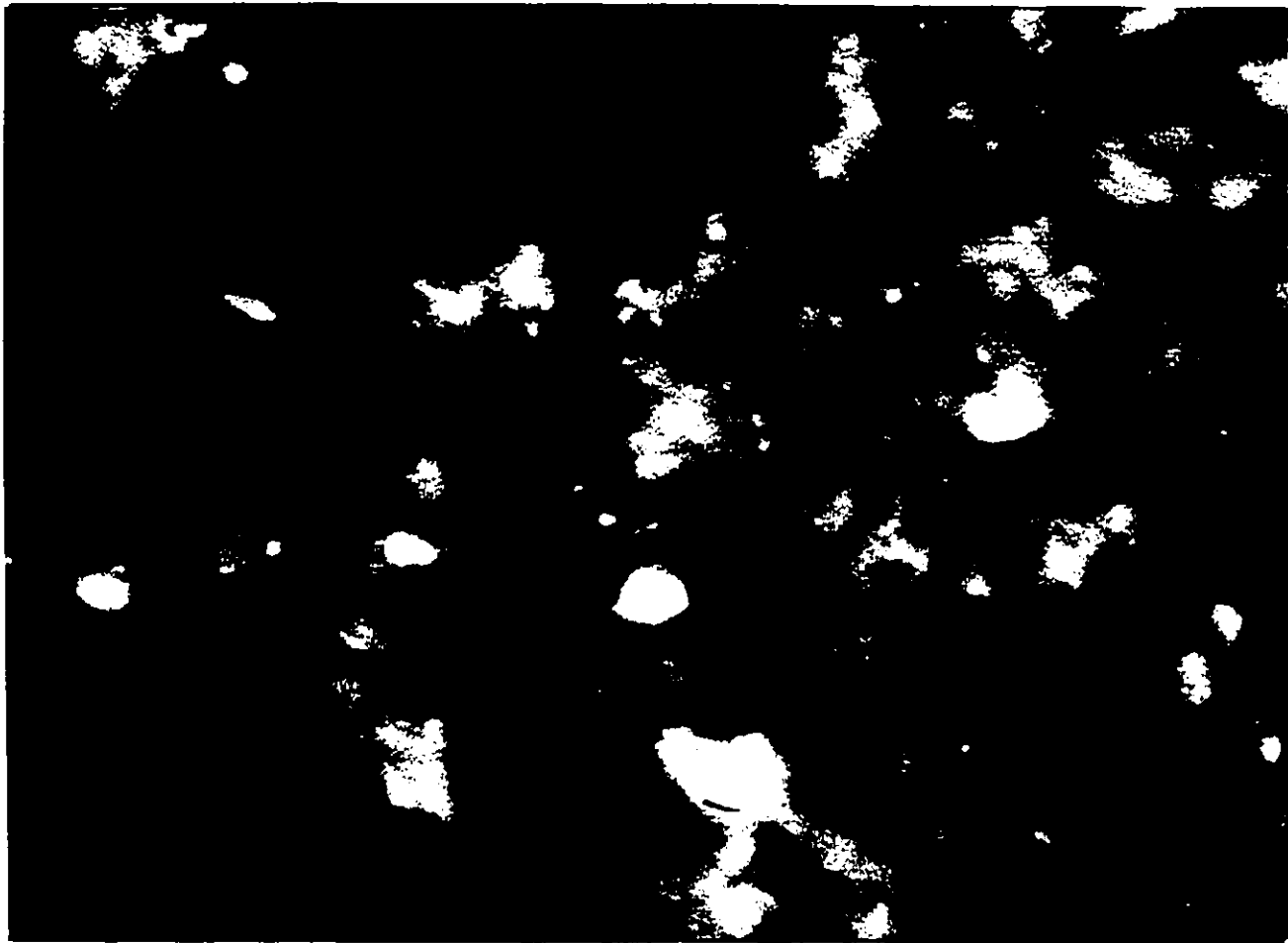
D. Hepatitis B Core Antigen (HB_cAg)

1. Liver

a) Microscopy:--Liver sections from an immunosuppressed renal transplant recipient with chronic hepatitis B antigenemia were incubated with fluorescein-conjugated anti-HB_c, anti-HB_s and anti-IgG (courtesy of Dr. S.N. Huang, McGill University, Montreal) and examined by phase contrast and fluorescent microscopy. Strong fluorescence was seen in approximately 35% of the hepatocytic nuclei following incubation with anti-HB_c (figures 30 and 31), with some granular fluorescence in the cytoplasm of a few scattered hepatocytes. Nuclear and cytoplasmic fluorescence were markedly increased

Figures 30 and 30a

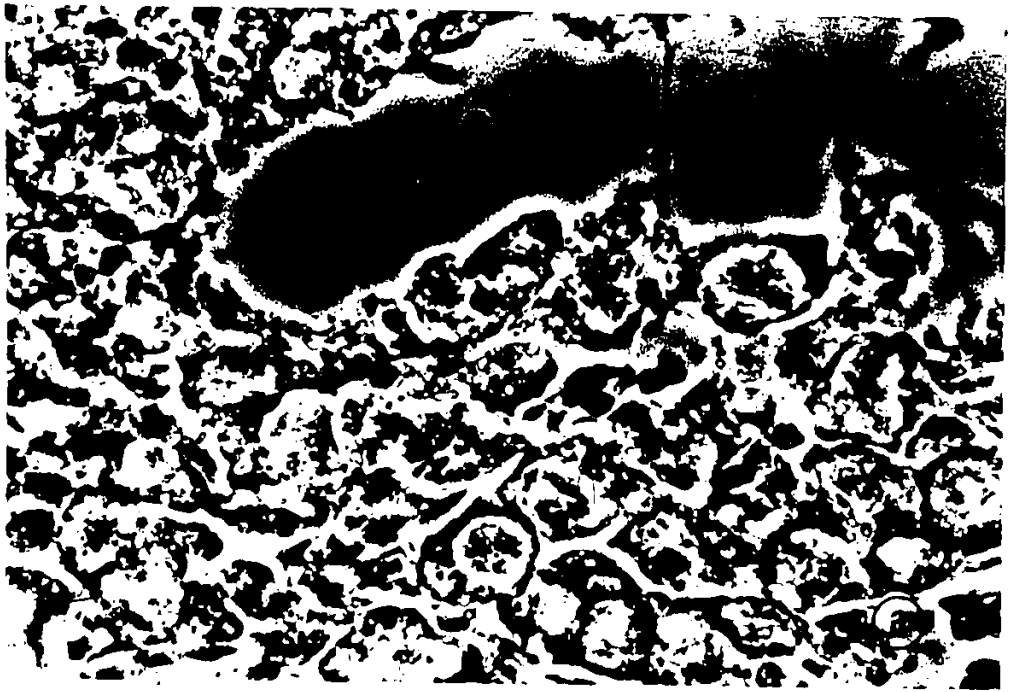
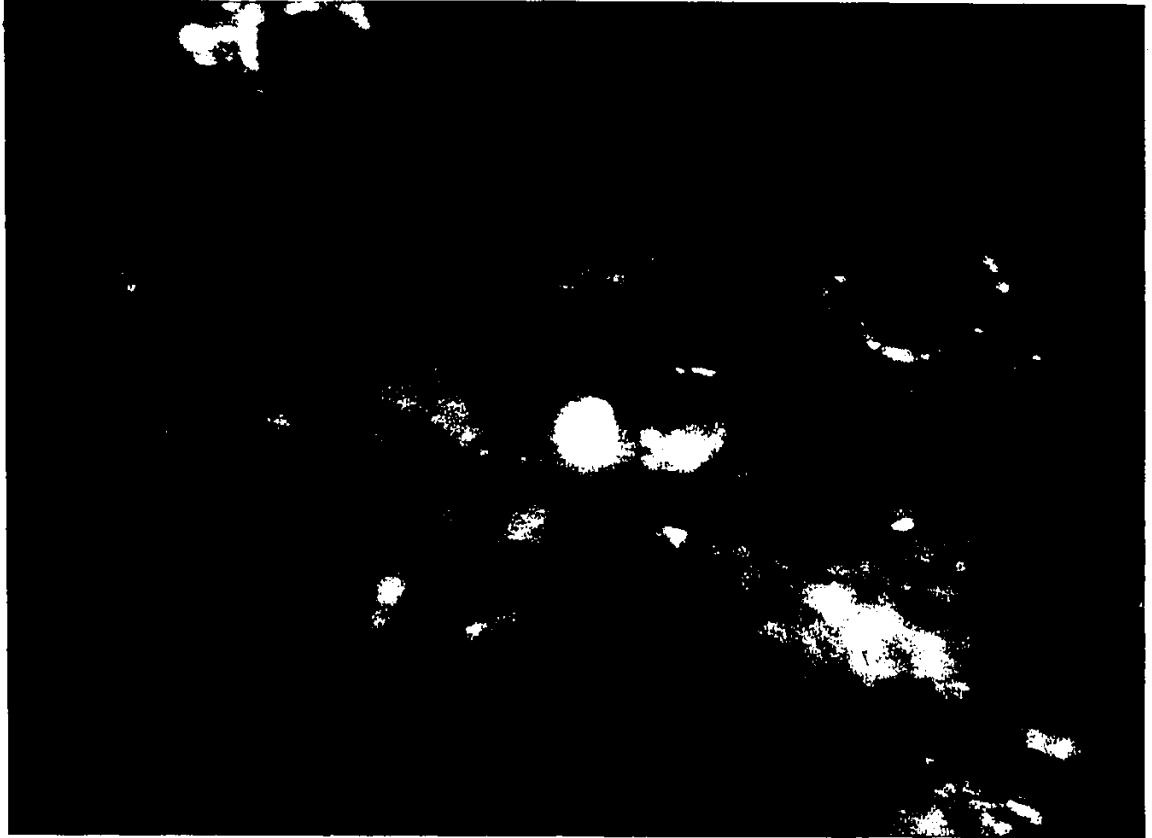
30. Immunofluorescence of hepatic nuclei from an HB_sAg-positive patient following incubation with fluorescein-conjugated anti-HB_c (1250 X).
- 30a. Phase contrast microscopy of the liver section showing intact hepatocytes (1250 X).



Figures 31 and 31a

31. Immunofluorescence of hepatic nuclei from an HB_sAg-positive patient following incubation with fluorescein-conjugated anti-HB_c (1250 X).

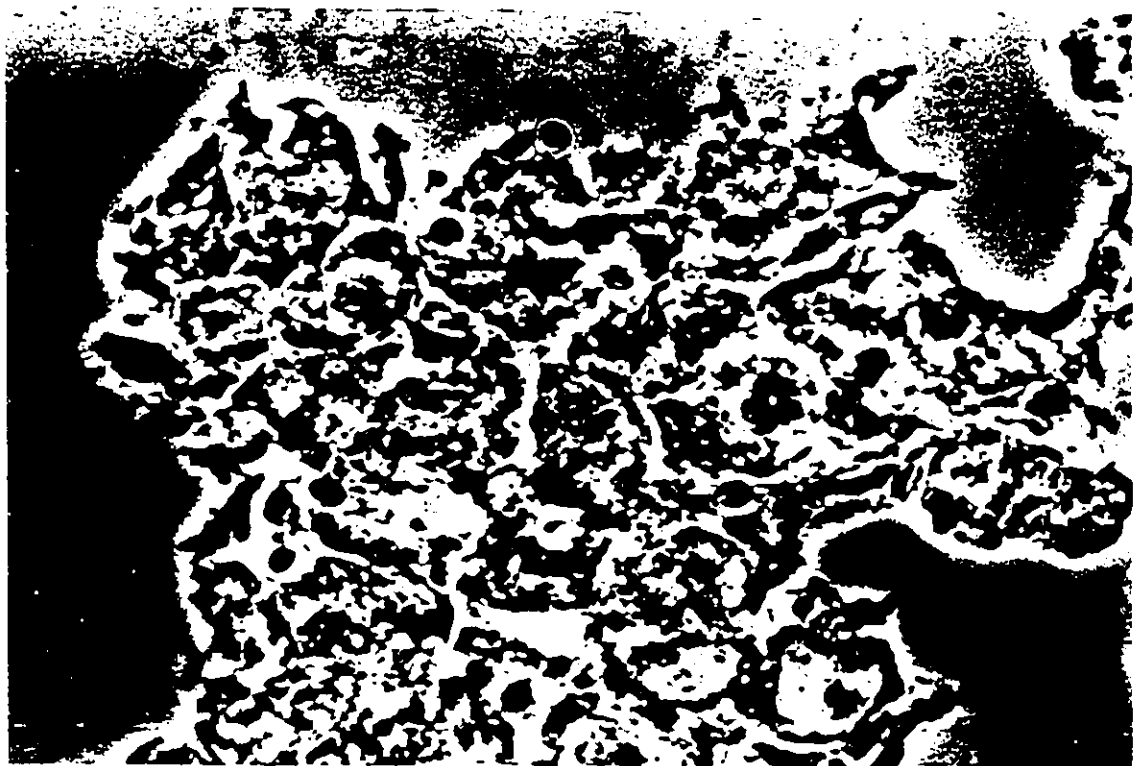
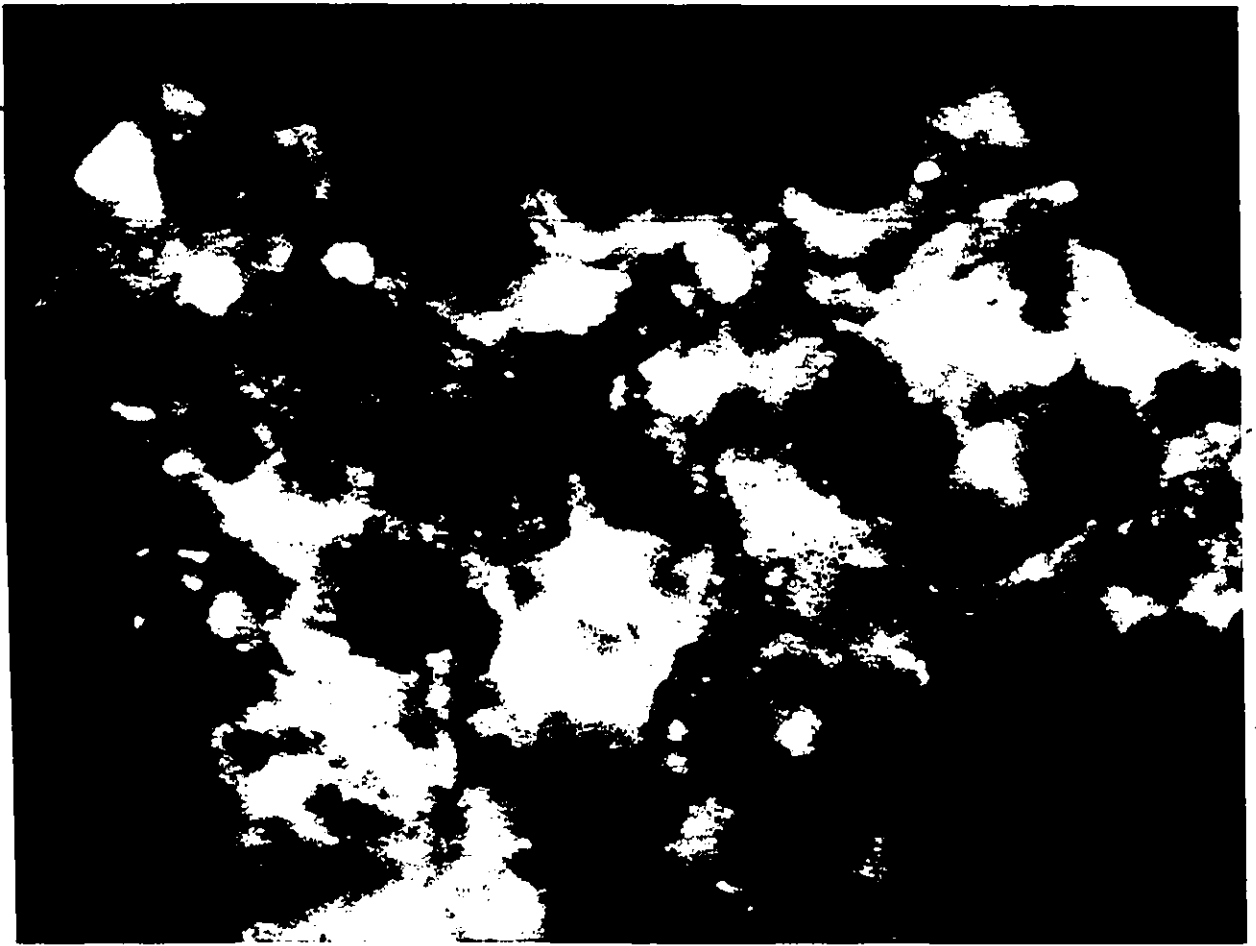
31a. Phase contrast microscopy of the liver section showing intact hepatocytes (1250 X).



Figures 32 and 32a

32. Immunofluorescence of hepatic nuclei from an HB_sAg-positive patient following incubation with fluorescein-conjugated anti-IgG (1250 X).

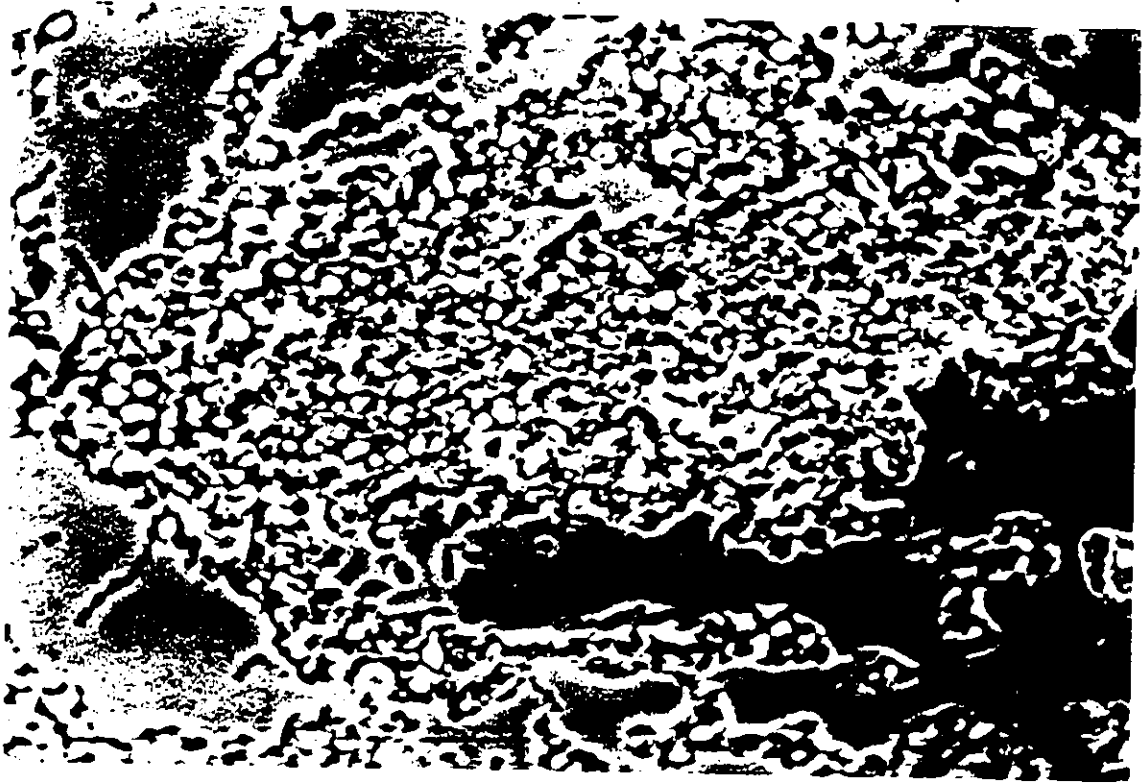
32a. Phase contrast microscopy (1250 X).



Figures 33 and 33a

33. Absence of hepatocyte immunofluorescence when incubated with fluorescein-conjugated anti-HB_s (300 X).

33a. Phase contrast microscopy (300 X).



in sections incubated with anti-IgG (figure 32). Incubation with anti-HB_s, on the other hand, yielded no detectable nuclear fluorescence and only a rare hepatocyte with cytoplasmic fluorescence (figure 33). Examination of the sections by phase contrast microscopy showed the hepatocytic architecture to be intact and well organized (figures 30a, 31a, 32a, 33a).

b) Purification of HB_cAg:--No spherical particles were seen by EM in the supernatant fluids and low-speed pellets discarded during the preparation of the nuclear pellets. Examination of this final pellet by phase contrast microscopy showed the nuclear membranes to be intact, with no adhering cytoplasm detectable.

Following disruption of the nuclei by sonication, the nuclear debris was pelleted at low speed and discarded. The supernatant was pelleted at high speed yielding a thick gelatinous pellet containing many spherical particles in clumps (figure 34). This pellet, however, was difficult to disrupt. As a result of this occurrence, it was decided to pellet the supernatant onto a 45% w/w sucrose cushion prior to isopycnic banding in CsCl. This added procedure greatly increased the yield of non-aggregated particles.

The isolated HB_cAg particles were recovered at a CsCl density of 1.30 g/cm³. Maximal RIA activity coincided with ultraviolet absorption ranging between 260-265 nm. Following dialysis against PBS, the particles were examined by electron microscopy and found to be spherical in shape with a mean diameter of 27 nm. Negative staining with 2%



Figure 34: Clump of 27 nm HB_cAg particles following high speed centrifugation

Bar represents 100 μ .

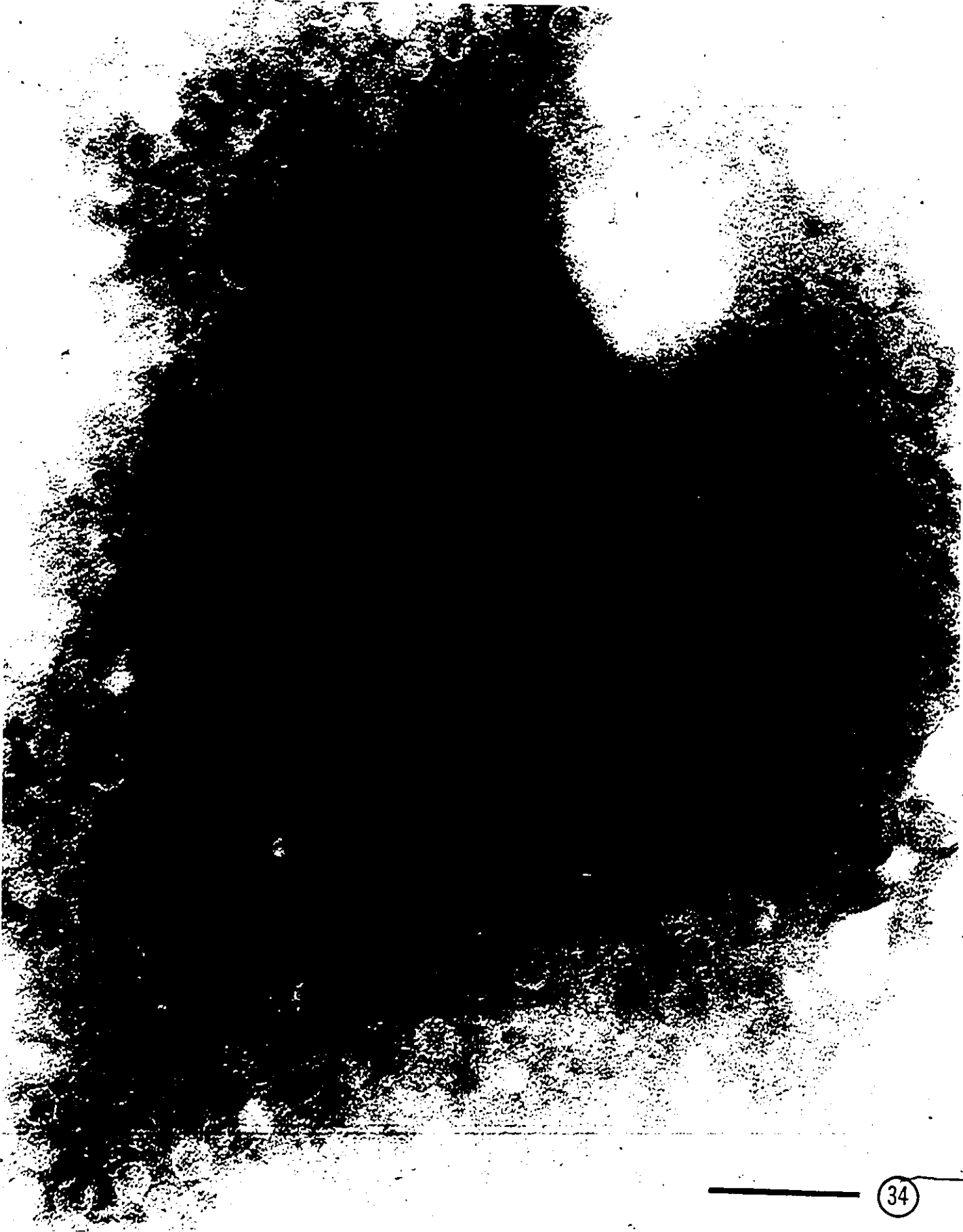
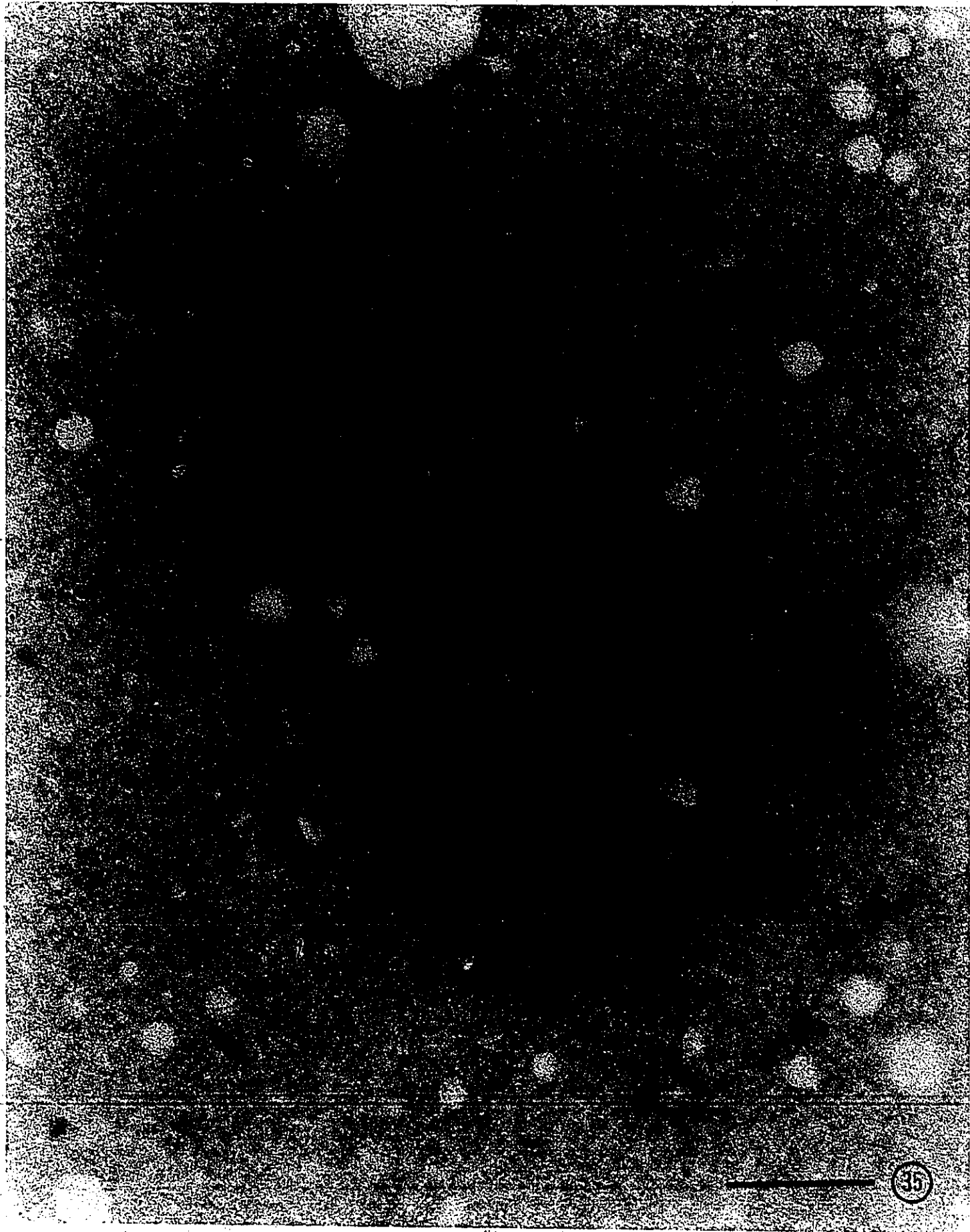




Figure 35: .27 nm HB_cAg particles following high speed pelleting onto a sucrose cushion.

Bar represents 100 μ.





Figures 36-39: Immune electron microscopy (IEM) of HB_cAg particles following incubation with anti-HB_c. Note the fine spicules between the 27 nm particles which are anti-HB_c.

Bar represents 100 μ .



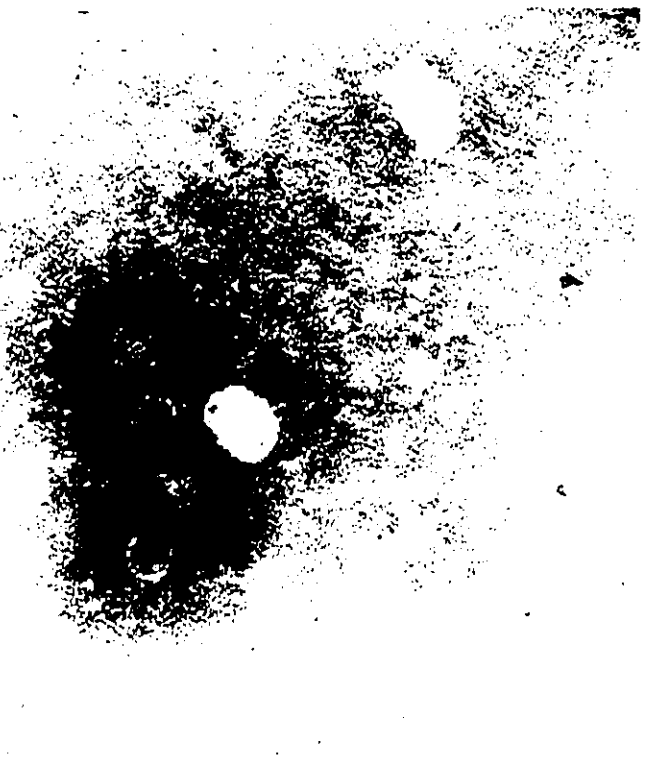
36



37



38



39

PTA showed that almost all particles were empty (figure 35).

No HB_sAg was detected by RIA in the fractions containing the 27 nm particles. Furthermore, incubation with anti-HB_s did not agglutinate these particles. In sharp contrast, incubation with anti-HB_c resulted in particle agglutination, with spike-like projections seen between the individual particles of the clusters examined (figures 36, 37, 38 and 39).

2. Serum

a) Dane particle purification:--Crude pellet preparations rich in Dane particles were selected for further purification by 2 rate separations on sucrose gradients as previously described. Whereas 20 nm spherical particles were recovered in fractions 13 - 17 (figure 21), Dane particles were found most often in fractions 4 - 6 (figure 40). Two populations of Dane particles were seen, designated as empty and full, based on their appearance following negative staining with 2% PTA (figures 41 and 42a,b). Following isopycnic banding in CsCl, these empty and full Dane particles were recovered at 1.20 and 1.25 g/cm³ respectively.

Fractions 7 - 17 from the 2nd rate separation banding contained decreasing numbers of filaments of varying lengths and increasing numbers of 20 nm particles as the sucrose density of the collected fractions decreased (figures 43, 44, 45 and 46).

b) Core extraction:--The Dane-rich fractions were pooled, detergent treated, passed through a rate separation

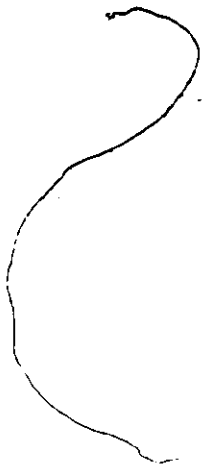


Figure 40: The rate separation banding of the pellet obtained at 21,000 rpm from HB_s Ag-positive sera prior to the large and small volume purification procedure. These pellets were from sera which were HB_e Ag and DNA polymerase positive.

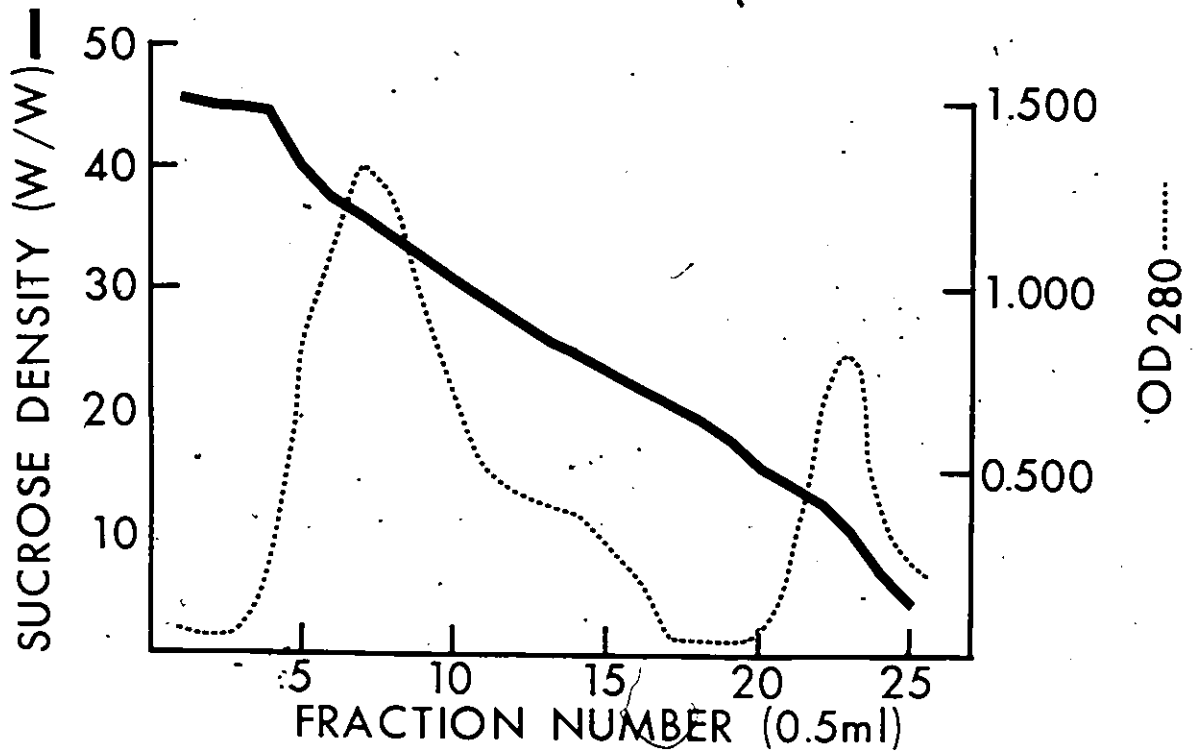
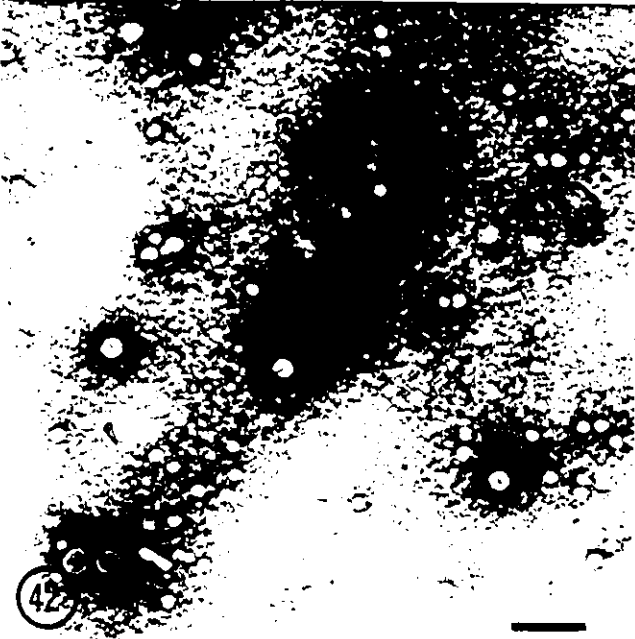
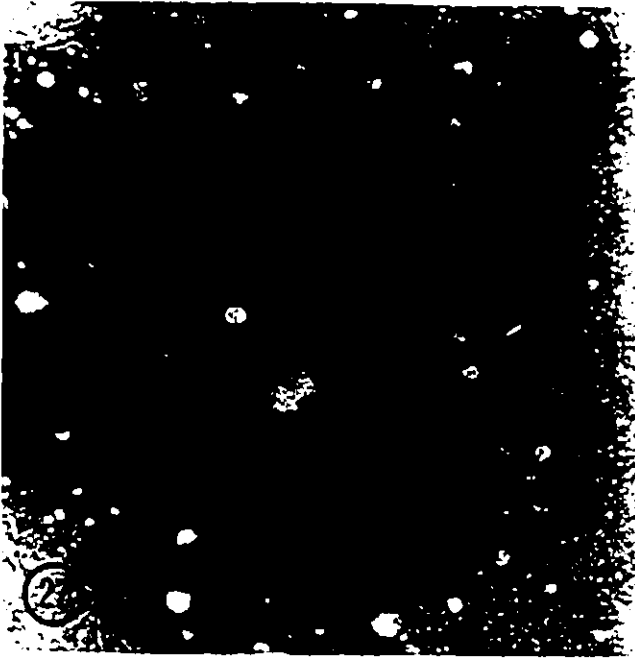


Figure 40

Figures 27, 41 a and b

27. A pellet from a DNA polymerase/HB_e Ag-positive serum showing both populations of Dane particles, empty and full. There are also filaments and 22 nm spherical particles present in this pellet.
- 41,) Full and empty Dane particles purified from three
42 a,b.) different DNA polymerase/HB_e Ag-positive sera.

All bars represent 100 μ .

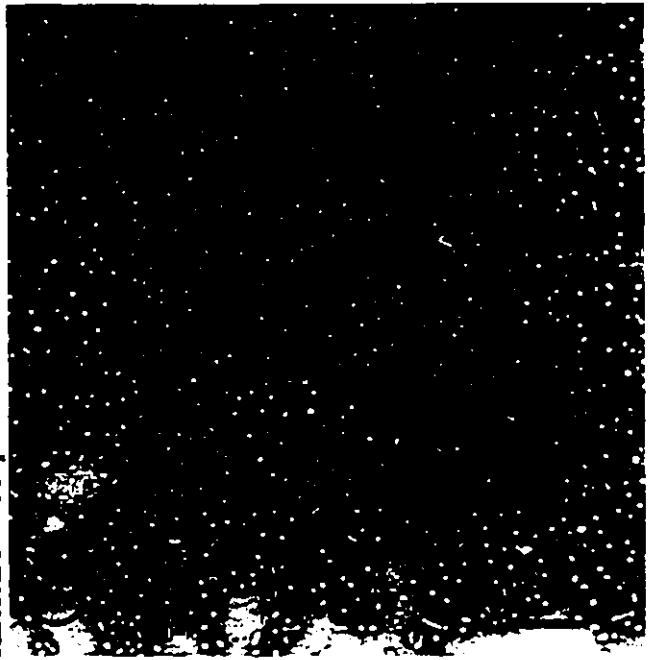
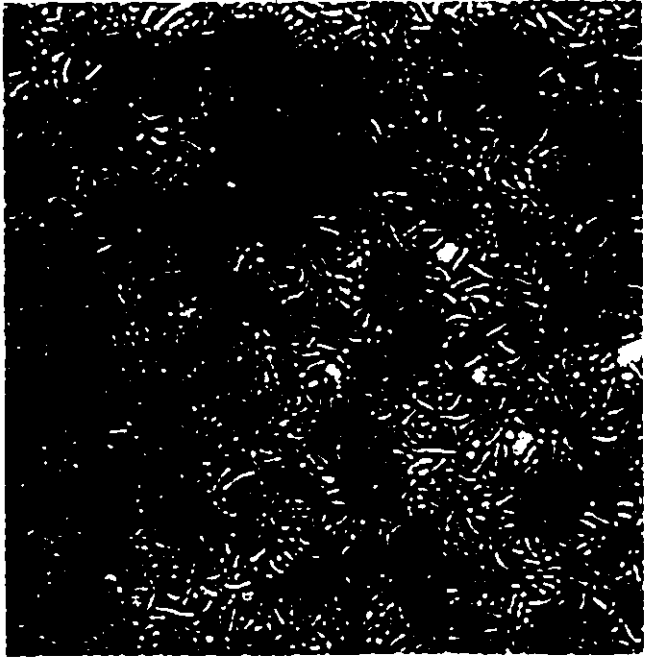
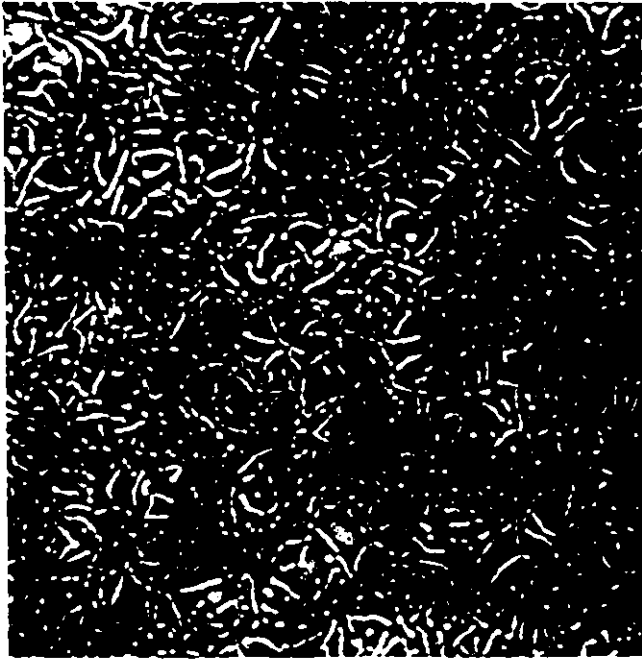


Figures 45-46

Four HB_sAg-positive fractions collected at four different densities in a sucrose gradient following the rate separation banding of the pellet recovered after a 21,000 rpm, 4 hour centrifugation of HB_sAg-positive serum.

43. Fraction 8. Long filaments with some empty Dane particles.
44. Fraction 11. Filaments are slightly shorter. An occasional Dane particle is still evident.
45. Fraction 13. Filament lengths have further decreased. Some 22 nm spherical particles are present.
46. Fraction 15. The majority of particles are 22 nm spherical particles with some very short filaments still present.

All bars represent 200 μ .



centrifugation step and banded isopycally. Two populations of HB_CAg were collected--empty and full--at 1.30 and 1.35 g/cm³ in CsCl. Neither of these two populations had detectable HB_SAg by RIA, nor were they agglutinated by anti-HB_S. These particles were agglutinated with anti-HB_C, as previously described.

A DNA polymerase assay was attempted on only one sample of each population because of the limited amount of material available. DNA polymerase activity was detected in the denser, "full" core sample (\bar{x} = 216 cpm) but not in the less dense "empty" HB_CAg sample (\bar{x} = 37 cpm).

II. Cell Mediated Immunity

A. Lymphocyte Stimulation Parameters

1. Serum supplements

Control cultures from HB_SAg-negative individuals were incubated in autologous, homologous and heterologous sera as described in Materials and Methods and the effects of these supplements were measured by tritiated thymidine (³H-T) uptake over a six day period. ³H-T incorporation by whole blood cultures did not differ significantly from day 1 to day 6. Similarly, cultures incubated in homologous (pooled AB Rh-negative) sera also resulted in only minimal increases in ³H-T uptake during the observation period. Cells incubated in heterologous (fetal bovine) sera, however, had markedly increasing ³H-T uptake each day over the six day period, with counts per minute (cpm) at day 6 often greater than 20,000 (figure 47).



Figures 47 and 48

47. Tritiated thymidine incorporation by whole blood cultures and by lymphocytes incubated in homologous or heterologous sera.
48. PHA stimulation of whole blood cultures and lymphocytes incubated in homologous or heterologous sera.

2. Mitogen stimulation

Cultures were incubated with autologous, homologous and heterologous sera and challenged with 10 µg phytohaemagglutinin (PHA), for one to six days. Optimal stimulation occurred at day 2 for the three culture systems, declining thereafter for the remainder of the observation period (figure 48). Stimulation indices (SI) exceeded 150 at day 2 for all three culture systems, and remained near or slightly above 100 at day 6 for the autologous and homologous sera cultures. In sharp contrast, the SI decreased rapidly for cells cultured in heterologous sera, and was often below 10 in 6 day cultures (figure 49).

3. Cell viability as a function of pH

The viability of all control cultures incubated at pH 7.4 (± 0.1) over a six day period was consistently greater than 90% by the trypan blue exclusion test, regardless of serum supplements used. Decreasing or increasing the pH below or above this level always resulted in a fall in cell viability, with 6 day viability levels at pH 8.2 and 6.6 (± 0.1 for each) at less than 40% and 20% respectively (figure 50a).

4. Lymphocyte stimulation as a function of age of individuals tested

Lymphocytes from individuals of varying age were challenged in vitro with PHA as previously described (figure 50b). Maximum stimulation occurred at day 2 for all age groups, with the highest cpm's noted in the 15 to 30 year old group

Figures 49, 50a and 50b

49. Stimulation indices of whole blood cultures and lymphocytes incubated in homologous or heterologous sera.
- 50a. Control lymphocyte cultures — viability as a function of pH.
- 50b. PHA-stimulated lymphocyte cultures — stimulation as a function of age.

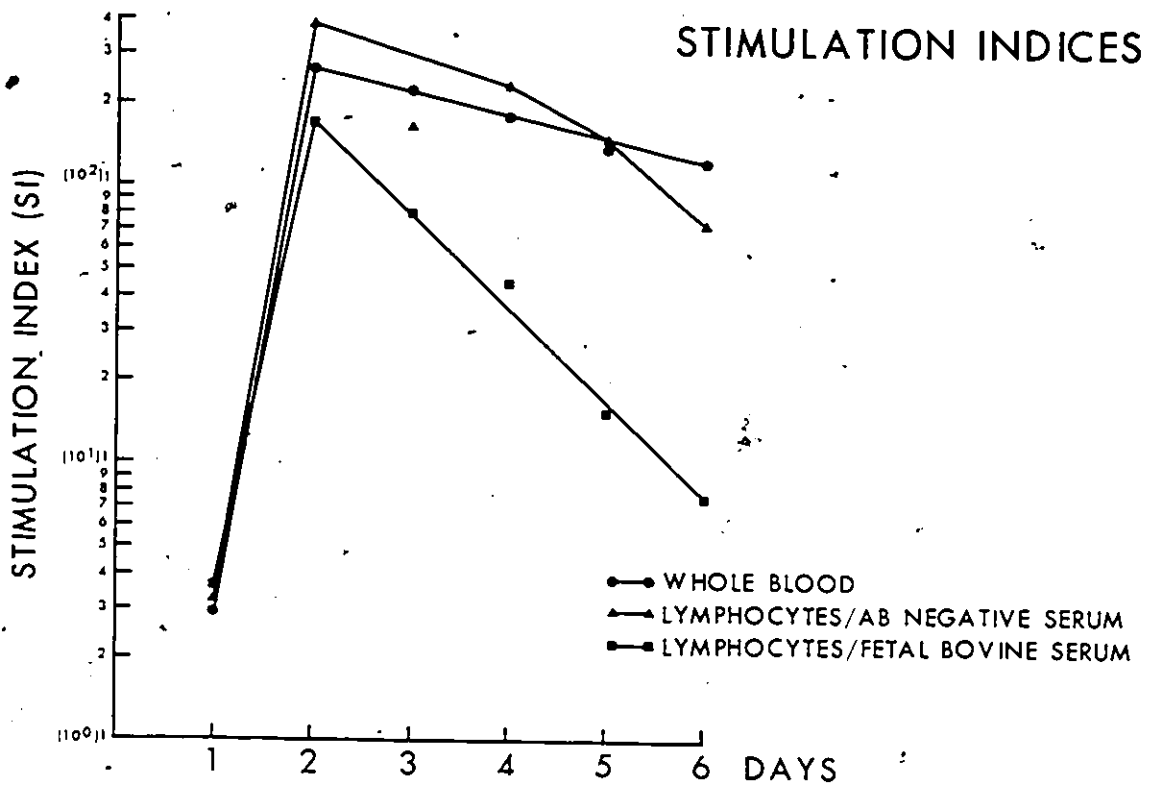


Figure 49

CONTROL LYMPHOCYTE CULTURES VIABILITY AS A FUNCTION OF pH

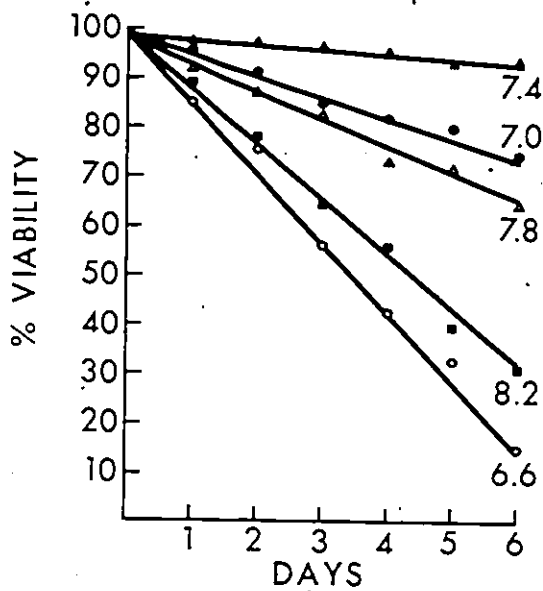


Figure 50a

PHA-STIMULATED LYMPHOCYTE CULTURES STIMULATION AS A FUNCTION OF AGE

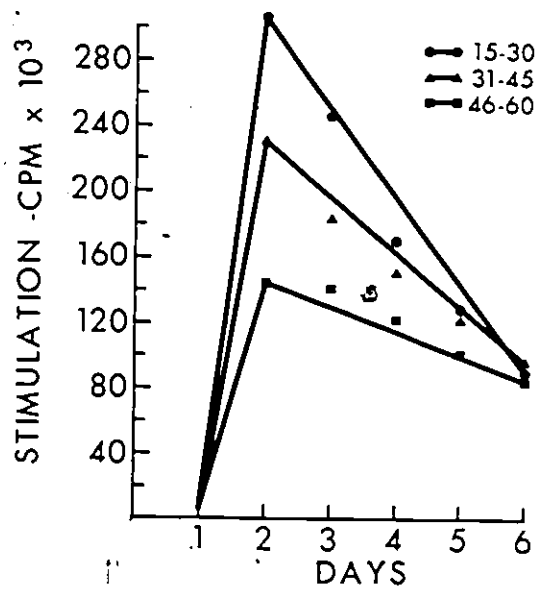


Figure 50b

(mean cpm approximately 300,000 for six individuals tested) and the lowest in those individuals between 40 and 60 years of age (mean cpm approximately 140,000). Stimulation declined in all groups after day 2, with mean cpm's for all groups approximating each other at day 6 (about 100,000 cpm).

B. Experiment I

1. Lymphocyte stimulation

Six subjects who had recovered from hepatitis B were selected. Two of the six had only recently cleared HB_sAg (subjects 3 and 4) and were anti-HB_s-negative at the time of study. Cell cultures were divided into three groups depending on the serum supplements added and were incubated with either 2, 5 or 25 µg of purified HB_sAg for a six day period. In none of the three groups were any significant stimulation indices (SI.) obtained (Table XXVa). The two anti-HB_s negative individuals had stimulation indices greater than 2 when incubated in AB-negative serum and challenged with 5 µg of purified HB_sAg; these results were not reproducible with the two other serum supplements. The S.I. of whole blood cultures were less than 1 when incubated with 2 µg of purified antigen. At higher antigen concentrations, however, the stimulation indices were consistently greater than 1. None of the normal control groups demonstrated these stimulation index variations following antigen challenge (Table XXVb). The results of the six post-hepatitis B subjects and the six normal controls are

TABLE XXVa

EXPERIMENT I. LYMPHOCYTE STIMULATION AND MIGRATION INHIBITION STUDIES ON POST
HEPATITIS B SUBJECTS AND MATCHED NORMAL CONTROLS

Subject Number (Sex;Age)	Interval (wks) since HB _s Ag Positive	Anti-HB _s	Unstimulated Control Cultures (cpm)	Lymphocyte Stimulation (S.I.)			Migration Inhibition (M.I.)		
				2μg HB _s Ag concentration	5μg HB _s Ag concentration	25μg HB _s Ag concentration	80μg HB _s Ag concentration	40μg HB _s Ag concentration	
1 (F;37)	50	+	270	1.01	0.90	1.10			
2 (F;31)	72	+	327	0.86	1.11	1.15			
3 (F;21)	2	-	340	0.72	1.37	1.28			
4 (F;23)	1	-	285	0.75	1.75	1.36			
5 (M;29)	22	+	329	0.95	1.25	1.05			
6 (M;24)	30	+	313	N/T	N/T	0.74			
				MEANS ± S.D. 311 ± 0.27 0.86 ± 0.13 1.28 ± 0.32 1.11 ± 0.22					
Post HB _s Ag--Whole Blood Cultures									
1 (F;37)	50	+	975	0.99	1.02	1.13	1.00	0.97	
2 (F;31)	72	+	1430	1.06	1.23	1.03	0.98	0.92	
3 (F;21)	2	-	1725	1.55	2.01	1.75	0.75	0.83	
4 (F;23)	1	-	1160	1.49	2.75	1.99	0.62	0.69	
5 (M;29)	22	+	830	1.15	1.26	1.20	0.85	0.90	
6 (M;24)	30	+	725	1.27	1.25	1.22	0.77	0.86	
				MEANS ± S.D. 1141 ± 380 1.25 ± 0.23 1.59 ± 0.65 1.39 ± 0.39 0.83 ± 0.15 0.86 ± 0.10					
Post HB _s Ag-AB Negative Sera Pool									

...Continued

TABLE XXVa
Continued

Subject Number (Sex; Age)	Interval (wks) since HB _s Ag Positive	Anti- HB _s	Unstimulated Control Cultures (cpm)	Lymphocyte Stimulation (S.I.)			Migration Inhibition (M.I.)	
				HB _s Ag concentration			HB _s Ag concentration	
				2μg	5μg	25μg	80μg	40μg
1 (F; 37)	50	+	17,485	0.89	1.08	1.40		
2 (F; 31)	72	+	22,680	0.96	1.17	1.33		
3 (F; 21)	2	-	33,250	1.05	0.96	1.18		
4 (F; 23)	1	-	26,195	1.10	1.12	1.05		
5 (M; 29)	22	+	19,845	1.03	1.25	0.92		
6 (M; 24)	30	+	21,455	1.22	1.03	1.25		
MEANS ± S.D.			23,485 ± 5,601	1.04 ± 0.12	1.10 ± 0.10	1.19 ± 0.18		

N/T--Not tested.

TABLE XXVb

EXPERIMENT I. LYMPHOCYTE STIMULATION AND MIGRATION INHIBITION STUDIES ON POST
HEPATITIS B SUBJECTS AND MATCHED NORMAL CONTROLS

Subject Number (Sex;Age)	Interval (wks) since HB _s Ag Positive	Anti-HB _s	Unstimulated Control Cultures (cpm)		Lymphocyte Stimulation (S.I.) HB _s Ag concentration			Migration Inhibition (M.I.) HB _s Ag concentration	
					2μg	5μg	25μg	80μg	40μg
7 (F;35)	N/A	-	267	0.87	0.99	0.74	0.98	1.07	1.00
8 (F;30)	N/A	-	239	1.27	1.55	1.25	1.31	0.71	0.78
9 (F;21)	N/A	-	316	1.29	1.16	1.09	1.12	1.05	0.89
10 (F;21)	N/A	-	284	0.89	1.30	1.51	0.81	1.00	0.87
11 (M;30)	N/A	-	325	1.11	1.04	1.16	0.75	0.96	1.03
12 (M;25)	N/A	-	278	1.03	1.25	1.05	1.23	0.82	0.93
			MEANS ± S.D.	285 ± 32	1.08 ± 0.18	1.22 ± 0.20	1.13 ± 0.25	0.94 ± 0.14	0.92 ± 0.09
<u>Normal Controls--Whole Blood Cultures</u>									
<u>Normal Controls--AB Negative Sera Pool</u>									
7 (F;35)	N/A	-	606	0.96	0.77	0.98	1.07	1.00	
8 (F;30)	N/A	-	996	1.76	1.37	1.31	0.71	0.78	
9 (F;21)	N/A	-	925	1.27	1.19	1.12	1.05	0.89	
10 (F;21)	N/A	-	816	1.22	1.22	0.81	1.00	0.87	
11 (M;30)	N/A	-	1435	1.09	1.04	0.75	0.96	1.03	
12 (M;25)	N/A	-	1178	1.05	0.93	1.23	0.82	0.93	
			MEANS ± S.D.	993 ± 289	1.23 ± 0.29	1.09 ± 0.22	1.03 ± 0.23	0.94 ± 0.14	0.92 ± 0.09

...Continued

TABLE XXVb
Continued

Subject Number (Sex;Age)	Interval (wks) since HBsAg Positive	Anti-HBs	Unstimulated Control Cultures (cpm)	Lymphocyte Stimulation (S.I.) HBsAg concentration			Migration Inhibition (M.I.) HBsAg concentration		
				2µg	5µg	25µg	80µg	40µg	
7 (F;35)	N/A	-	19,325	1.38	1.06	1.12			
8 (F;30)	N/A	-	24,240	1.08	1.22	1.37			
9 (F;21)	N/A	-	31,455	1.22	1.11	1.10			
10 (F;21)	N/A	-	27,210	0.91	1.00	1.22			
11 (M;30)	N/A	-	19,885	1.04	0.95	1.00			
12 (M;25)	N/A	-	21,105	0.85	0.90	0.82			
MEANS ± S.D.				23,870 ± 4,779	1.08 ± 0.20	1.04 ± 0.12	1.11 ± 0.19		

Normal Controls--Fetal Bovine Sera Pool

N/A--Not applicable.

- Figures 51 and 52

51. Tritiated thymidine incorporation by whole blood cultures and lymphocytes cultured in homologous and heterologous serum following incubation with three different purified HB_sAg concentrations.
52. Stimulation indices* following pre-incubation with four different concentrations of purified HB_sAg.

* (Indices less than 0.80 and greater than 1.20 constitute significant inhibition and migration respectively).

CULTURE TECHNIQUES

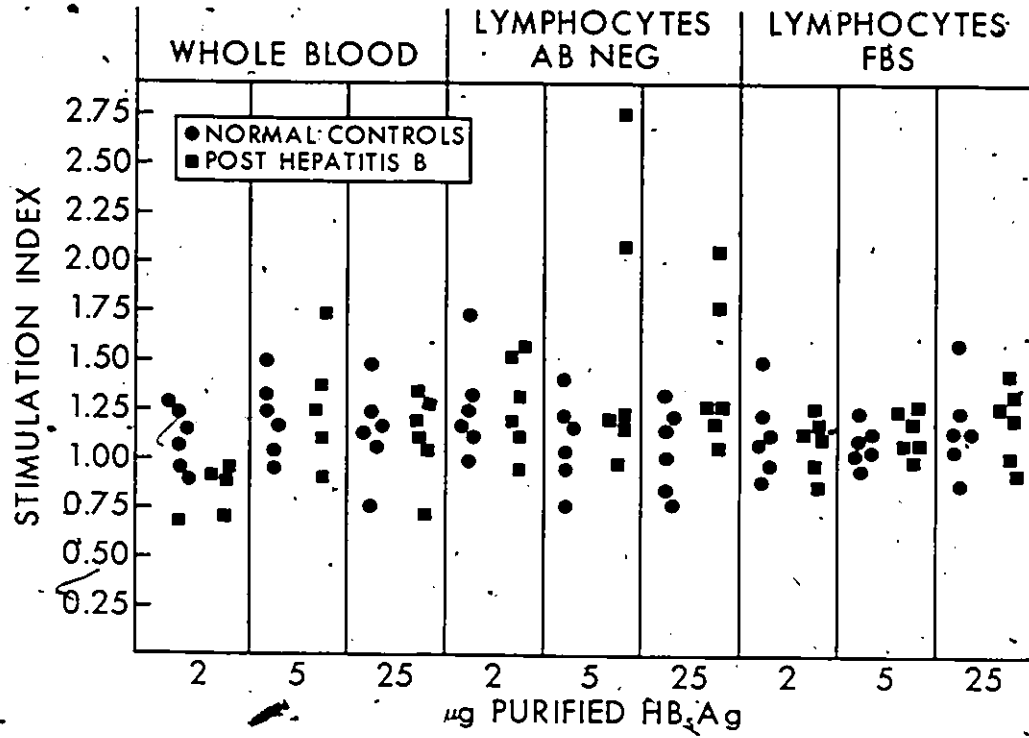


Figure 51

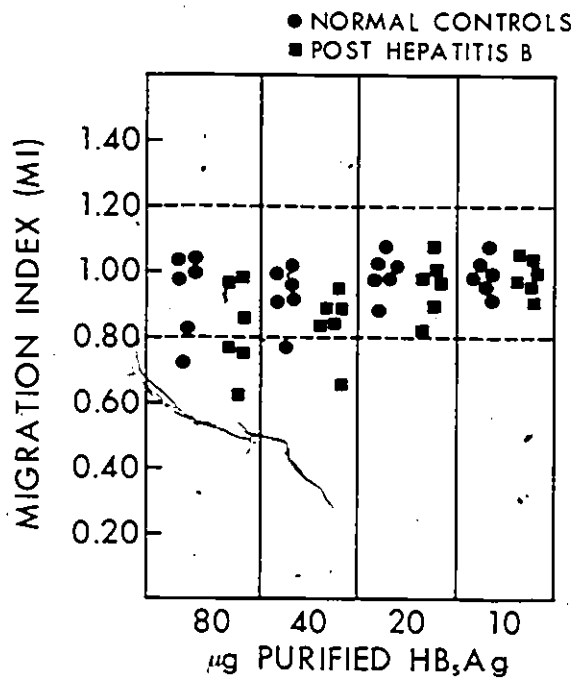


Figure 52

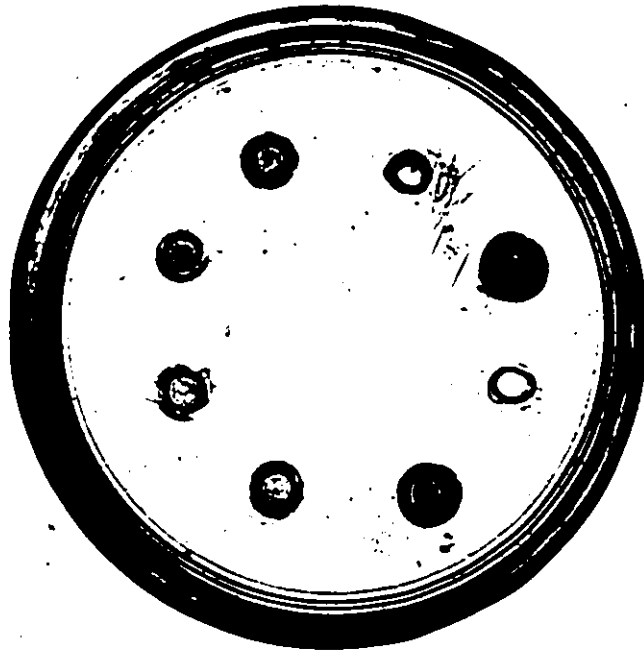
graphically compared in figure 51.

All twelve subjects tested were found to have normal white cell counts with normal differentials. Two day PHA-stimulated cultures performed concomitantly had stimulation indices greater than 200 in all groups (whole blood culture mean 273: range 184 - 491; AB negative serum mean 258: range 193 - 447; fetal bovine serum mean 207: range 143 - 338). Viability for all cultures by the trypan blue exclusion test was greater than 90%.

2. Migration inhibition

The migration inhibition test was carried out simultaneously with the lymphocyte stimulation study. Three of the six post-hepatitis B patients had migration indices (M.I.) of less than 0.80 following pre-incubation with 80 μ g of purified HB_sAg (table XXVa). Included in this number were the two anti-HB_s negative individuals. Pre-incubation with 40 μ g HB_sAg reduced this number to 1. At lesser antigenic concentrations (i.e., 20 and 10 μ g), all indices were greater than 0.80. By contrast, only one of the six normal controls had an M.I. less than 0.8 when pre-incubated with 80 μ g of the purified antigen; however, a similar inhibition was also obtained with 40 μ g (table XXVb). The means for the post-hepatitis B and normal controls were both greater than 0.80 and the difference between the two groups was not statistically significant. The migration indices of these two groups are graphically compared in figure 52.

Figure 53: An agarose plate showing migration inhibition. The wells with the wider rims contain leukocytes pre-incubated in TC 199 while leukocytes pre-incubated with 80 μ g of purified HB_sAg show only a narrow rim after staining with Wright's stain. The two wells with no staining contain saline. More than 98% of the migrating leukocytes are polymorphonuclear cells.



53

80

S

80

C

80

S

80

C

3. Identification of the migrating cells

Following measurement of the areas of cellular migration by planimetry, the plates were fixed and stained with Wright's stain (figure 53). Four hundred migrating cells were differentially counted per plate using a light microscope. More than 98% of all cells counted were polymorphonuclear leukocytes.

C. Experiment II

1. Lymphocyte stimulation (figure 54)

Ten anti-HB_s-positive subjects and 10 HB_sAg/anti-HB_s-negative controls were selected. Five of the ten anti-HB_s-positive patients had had acute hepatitis B 11 to 30 months earlier, while the remainder had no history of the illness. All twenty subjects had normal white cell counts with normal differentials. Lymphocyte cultures from the anti-HB_s-positive group showed no significant stimulation when incubated for six days with three different concentrations of either purified HB_sAg or HB_sAg vaccine (a formalin inactivated pool of purified HB_sAg). Incubation with purification contaminants on the other hand resulted in significant stimulation with Fraction 1 ($0.01 > p > 0.001$) but a decrease in ³H-thymidine uptake with fraction 4 ($0.05 > p > 0.02$). Stimulation indices with fractions 2 and 3 were not significant (Table XXVIa). Fraction 1 was negative for HB_sAg by CIEP but positive by RIA (P/N ratio of 8.4 at 10⁰ dilution) whereas fractions 2, 3 and 4 were negative by both techniques (see

TABLE XXVIA

EXPERIMENT II. LYMPHOCYTE STIMULATION STUDIES ON ANTI-HB_S POSITIVE SUBJECTS
(± PREVIOUS ACUTE HEPATITIS B ILLNESS) AND MATCHED NEGATIVE CONTROLS

Subject Number (Sex;Age)	Previous illness interval	Unstimulated controls (cpm)	Lymphocyte Stimulation Index													
			Purified HB _S Ag						HB Ag Vaccine						Purification contaminants	
			2µg	5µg	25µg	25µg	2µg	5µg	25µg	25µg	1	2	3	4		
<u>Anti-HB_S Positive*</u>																
1 (F;31)	+(26m)	899	1.68	1.19	1.49	1.33	1.25	1.29	4.29	2.12	1.29	0.42				
2 (F;22)	+(11m)	1209	0.72	0.70	0.96	0.88	0.85	0.75	1.48	1.33	1.08	0.36				
3 (F;27)	-	833	0.81	1.03	1.44	1.08	1.15	1.21	2.09	1.35	1.26	0.87				
4 (F;24)	+(15m)	1323	1.23	1.27	1.40	1.47	1.43	1.79	7.01	5.06	1.92	0.78				
5 (F;25)	-	1553	1.04	1.16	1.24	1.08	1.49	1.49	4.70	1.59	0.76	0.69				
6 (F;45)	-	1156	0.70	0.92	0.76	0.73	0.69	0.64	1.89	0.89	0.95	0.60				
7 (M;34)	+(28m)	770	1.43	1.49	1.06	0.85	1.28	1.08	6.08	1.95	0.84	0.65				
8 (M;21)	+(30m)	1056	0.47	0.72	0.75	0.85	0.92	0.80	1.74	1.23	1.82	0.49				
9 (M;32)	-	959	0.95	0.95	1.01	1.45	1.09	1.88	2.40	1.48	0.87	1.31				
10 (M;25)	-	836	1.11	0.89	0.85	0.97	1.04	0.88	4.51	2.02	1.51	1.26				
	Means±	1059±	1.01±	1.03±	1.10±	1.07±	1.12±	1.18±	3.62±	1.90±	1.23±	0.74±				
	S.D.	264	0.37	0.25	0.28	0.26	0.25	0.43	1.99 ¹	1.17	0.41	0.33 ²				

¹0.01 > p > 0.001 (Contaminant 1 vs control).

²0.05 > p > 0.02 (Contaminant 4 vs control).

figure 7 for fraction source).

Cultures from the ten negative controls also showed no significant stimulation when incubated with the two previously described antigen preparations. Purification contaminant 1 produced significant lymphocyte stimulation in this group as well ($0.02 > p > 0.01$) but unlike the anti-HB_s-positive group, fraction 4 did not decrease ³H-thymidine uptake (Table XXVIb).

Cell viability for all cultures for the two groups was greater than 90% by the trypanblue exclusion test. Two-day PHA cultures had mean S.I.'s of greater than 200 for both groups with cell viability consistently greater than 80%.

Normal human serum (NHS), negative for HB_s Ag and anti-HB_s, was treated in a method identical to that described for HB_s Ag serum purification to yield 4 contaminant fractions designated 1' to 4'. These were added to lymphocyte cultures of five individuals from each of the two groups. The resultant S.I. of fraction 1-stimulated anti-HB_s-positive cultures was 5.32 ± 1.18 but only 2.10 ± 0.57 when incubated with fraction 1' ($0.01 > p > 0.001$). The difference in ³H-thymidine uptake between fraction 1-stimulated anti-HB_s-positive cultures and those of the negative controls were similarly significant (Table XXVIc). None of the other fraction stimulation comparisons for the two groups were statistically different.

TABLE XXVIB

EXPERIMENT II. LYMPHOCYTE STIMULATION STUDIES ON ANTI-HB_S POSITIVE SUBJECTS
(± PREVIOUS ACUTE HEPATITIS B ILLNESS) AND MATCHED NEGATIVE CONTROLS

Subject Number (Sex;Age)	Previous illness interval	Unstimulated controls (cpm)	Lymphocyte Stimulation Index											
			Purified HB _S Ag				HB _S Ag Vaccine				Purification Contaminants			
			2µg	5µg	25µg	25µg	2µg	5µg	25µg	25µg	1	2	3	4
Negative Controls														
11 (F;30)	N/A	927	1.30	1.12	1.26	1.40	1.17	1.85	1.41	1.51	0.76	0.99		
12 (F;20)	N/A	1114	1.45	1.38	1.16	0.93	1.28	1.08	1.67	1.36	1.24	0.76		
13 (F;25)	N/A	1025	0.95	0.93	0.69	0.89	1.00	0.87	1.80	3.04	1.43	1.08		
14 (F;24)	N/A	1216	0.91	1.13	1.12	1.04	1.18	1.18	2.30	1.88	0.88	0.94		
15 (F;27)	N/A	847	1.14	0.88	0.76	1.35	1.40	1.35	1.95	1.99	1.12	1.54		
16 (F;42)	N/A	616	1.10	1.23	1.02	1.17	0.99	0.97	2.11	1.81	0.76	1.55		
17 (M;31)	N/A	1327	1.84	1.23	1.42	1.14	1.22	1.61	3.67	3.09	1.17	1.28		
18 (M;22)	N/A	1474	1.05	0.78	0.92	1.01	0.86	1.09	1.90	1.06	0.83	1.06		
19 (M;29)	N/A	1103	0.69	0.97	0.95	0.81	0.95	1.22	2.01	1.13	0.94	0.89		
20 (M;27)	N/A	955	0.75	1.01	1.09	0.85	0.89	1.13	1.55	0.95	1.03	1.31		
	Means±	1060±	1.12±	1.07±	1.04±	1.06±	1.09±	1.24±	2.04±	1.78±	1.02±	1.14±		
	S.D.	236	0.34	0.18	0.22	0.20	0.18	0.30	0.63 ¹	0.76	0.22	0.27		

N/A--Not applicable.

¹0.02 > p > 0.01 (Contaminant 1 vs control).

TABLE XXVIC
 EXPERIMENT II. LYMPHOCYTE STIMULATION STUDIES ON ANTI-HB_S POSITIVE SUBJECTS
 (± PREVIOUS ACUTE HEPATITIS B ILLNESS) AND MATCHED NEGATIVE CONTROLS

Subject Number (Sex;Age)	Previous Illness Interval	Lymphocyte Stimulation. (S.I.) Purification Contaminants							
		1	1'	2	2'	3	3'	4	4'
<u>Anti-HB_S Positive</u>									
1 (F;31)	+(26m)	4.29	2.16	2.12	1.39	1.29	1.36	0.42	1.22
4 (F;24)	+(15m)	7.01	1.99	5.06	2.04	1.92	1.41	0.78	1.03
5 (F;25)	-	4.70	1.75	1.59	1.54	0.76	1.27	0.69	0.91
7 (M;34)	+(28m)	6.08	3.02	1.95	1.08	0.84	1.39	0.65	1.02
10 (M;25)	-	4.51	1.56	2.02	1.75	1.51	0.96	1.26	1.07
Means ± S.D.		5.32±	2.10±	2.55±	1.56±	1.26±	1.28±	0.76±	1.05±
		1.181	0.571	1.42	0.36	0.48	0.18	0.31	0.11
<u>Negative Controls</u>									
11 (F;30)	N/A	1.41	1.35	1.51	1.39	0.76	1.30	0.99	1.16
14 (F;24)	N/A	2.30	1.26	1.88	0.96	0.88	1.06	0.94	1.05
15 (F;27)	N/A	1.95	1.55	1.99	1.36	1.12	0.97	1.54	1.12
17 (M;31)	N/A	3.67	1.67	3.09	1.57	1.17	0.85	1.28	1.26
20 (M;27)	N/A	1.55	1.90	0.95	1.26	1.03	1.05	1.31	1.09
Means ± S.D.		2.18±	1.55±	1.88±	1.31±	0.99±	1.05±	1.21±	1.14±
		0.921	0.25	0.79	0.23	0.17	0.16	0.25	0.08

N/A---Not applicable.

1 (anti-HB_S+) vs 1 (Neg. Cont.) } 0.01 > p > 0.001
 1 vs 1'

Figures 54 and 55

54. Tritiated thymidine incorporation by lymphocyte cultures following incubation with purified HB_sAg, HB_sAg vaccine and contaminants recovered during the purification procedure.
55. Stimulation indices following pre-incubation with four different concentrations of purified HB_sAg.

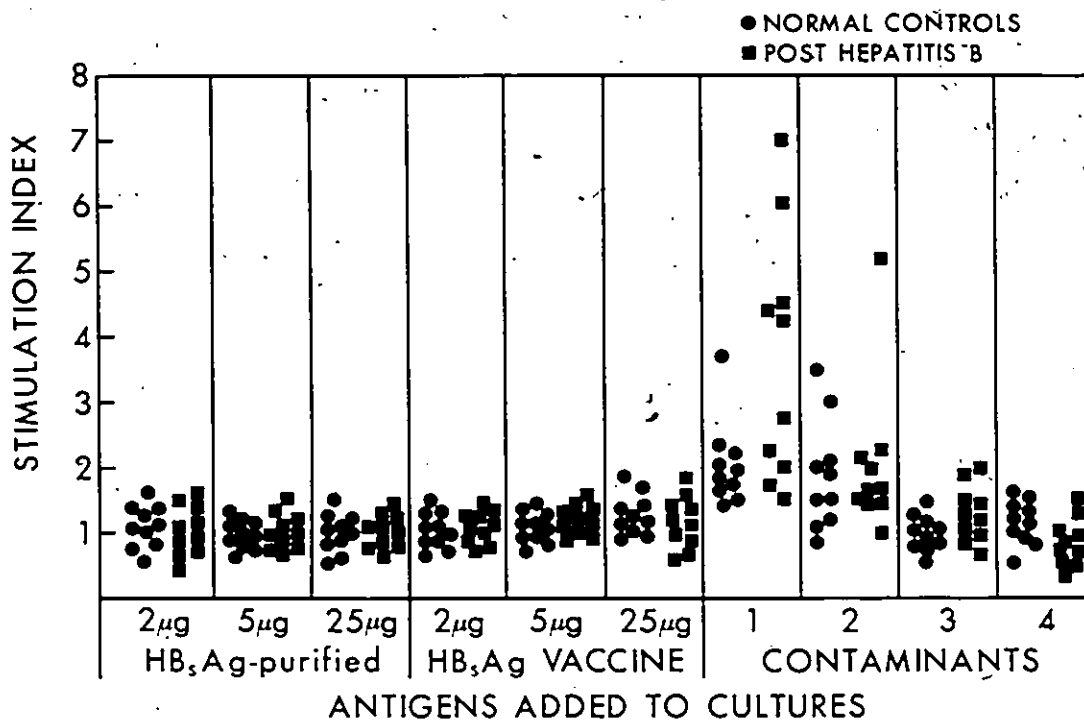


Figure 54

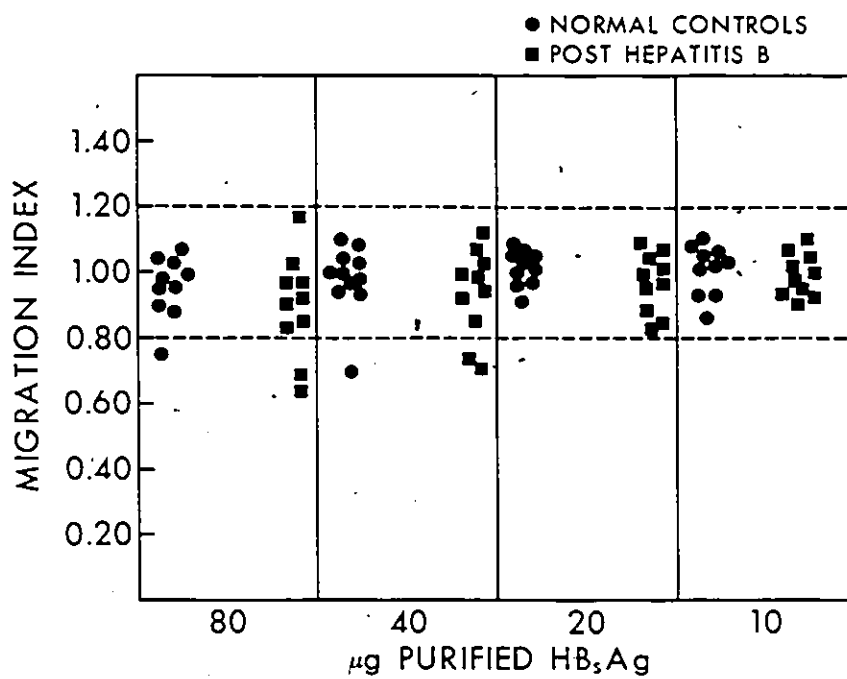


Figure 55

2. Migration inhibition

Two of the ten anti-HB_s-positive patients had M.I.'s of less than 0.80 with both 80 and 40 µg of purified HB_sAg. Neither of these two individuals had had acute hepatitis B previously. By comparison, one of the ten negative controls had an M.I. of less than 0.80 with these two antigenic concentrations (Table XXVId). The mean M.I.'s for the two groups, however, were all equal to or greater than 0.90 and the differences between them and the migration controls were not statistically significant. When leukocytes were pre-incubated with 20 and 10 µg of purified antigen, none of the individual M.I.'s were below 0.80 in either group (figure 55).

TABLE XXVII
 EXPERIMENT-II. MIGRATION INHIBITION STUDIES ON ANTI-HB_S
 POSITIVE SUBJECTS (\pm PREVIOUS ACUTE HEPATITIS B
 ILLNESS) AND ON MATCHED NEGATIVE CONTROLS

Subject Number (Sex; Age)	Previous Acute Illness	Migration Inhibition (M.I.) HB _S Ag concentration	
		80 μ g	40 μ g
<u>Anti-HB_S Positive</u>			
1 (F;31)	+	1.18	1.13
2 (F;22)	+	0.98	0.97
3 (F;27)	-	0.89	0.91
4 (F;24)	+	0.82	0.93
5 (F;25)	-	0.66	0.75
6 (F;45)	-	1.04	0.85
7 (M;34)	+	0.96	1.02
8 (M;21)	+	0.93	1.07
9 (M;32)	-	0.71	0.73
10 (M;25)	-	0.85	0.98
	MEANS \pm S.D.	0.90 \pm 0.15	0.93 \pm 0.12
<u>Negative Control</u>			
11 (F;30)	N/A	1.08	1.03
12 (F;20)	N/A	0.99	1.05
13 (F;25)	N/A	0.94	1.00
14 (F;24)	N/A	1.02	1.10
15 (F;27)	N/A	0.88	0.95
16 (F;42)	N/A	0.90	0.99
17 (M;31)	N/A	0.95	0.93
18 (M;27)	N/A	1.05	1.11
19 (M;29)	N/A	0.74	0.70
20 (M;27)	N/A	0.86	1.00
	MEANS \pm S.D.	0.94 \pm 0.10	0.99 \pm 0.12

N/A--Not applicable.

DISCUSSION

I. Hepatitis B Antigens and Antibodies

Progress in hepatitis B research was hampered until fairly recently by the lack of a specific serological assay. Following the discovery of HB_sAg, increasingly sensitive assays were developed which have, in large part, been responsible for our increased understanding of the disease and the remarkable advances that have occurred in recent years.

Among the most sensitive of the assays currently available is the RIA (Walsh et al, 1970). Comparative studies have consistently shown this assay to be more sensitive than either CIEP or AGD in identifying HB_sAg-positive sera (Aach et al, 1971; Hollinger et al, 1971; Ling and Overby, 1972; Giorgini et al, 1972; Hollinger et al, 1973) and on a par with the HA technique for detecting anti-HB_s (Peterson et al, 1973). Preliminary reports indicated that the RIA was 500 to 4,000 times as sensitive as CIEP for detecting HB_sAg in serum (Hacker and Aach, 1973; Cossart et al, 1973) and when used as a screening test in blood donors, could detect as many as ten times the number of positives detected by CIEP (Prince et al, 1973). Subsequent studies revealed that most of this apparent difference in sensitivity was due to the large number of false

positive reactions caused by the non-specific binding of test reagents with radiolabelled guinea pig anti-HB_s (Alter et al, 1972; Vyas et al, 1973; Purcell et al, 1973). Specificity of the test increased when ¹²⁵I-labelled human anti-HB_s was substituted for the guinea pig globulins (Aus RIA II test, Abbott Labs) but, as a result sensitivity decreased. Revised estimates of sensitivity indicated that RIA would detect only 2 to 3 times the number of true positives as CIEP (Irwin et al, 1974). Results from the present study confirmed that RIA is indeed superior in sensitivity to CIEP and AGD but the differences were much more modest, with only 5 to 20% more positives detected than with CIEP.

Evaluation of 195 consecutive sera from institutionalized Down's Syndrome (DS) patients and 2400 consecutive sera from patients with other forms of mental retardation (OMR) showed HB_s Ag to be 3 times as prevalent in the DS population. These findings were in agreement with those from other institutionalized populations (Szmunness et al, 1970; Szmunness and Prince, 1971; Hollinger et al, 1972; Ferris et al, 1972; Perry et al, 1975). They also showed that persistence of antigenemia was influenced by length of residence and age at admission, particularly in the DS group. The prevalence of HB_s Ag was higher in DS patients admitted in early childhood and persisted for a longer period of time. Unlike HB_s Ag, we found that anti-HB_s was equally detectable in both the DS and OMR populations and differed from results reported by other investigators (Szmunness and Prince, 1971; Szmunness

et al, 1972; Perry et al, 1975; Chaudhary et al, 1977). Our findings are in agreement with a recent study which showed no significant differences in anti-HB_s between institutionalized DS and OMR residents; furthermore, these investigators concluded that neither the length of residence nor the age at admission were significant factors in determining antigen clearance (Hawkes et al, 1980).

The hepatitis B surface antigen is antigenically heterogeneous. In addition to the group specific "a" determinant common to all HB_sAg-positive particles (Levene and Blumberg, 1969), there are also two subspecific determinants present - either "d" or "y" and "w" or "r" (Le Bouvier, 1971; Bancroft et al, 1972). Several investigators have independently identified other antigenic markers (Kim and Tilles, 1971; Gust, 1971; Magnus and Espmark, 1972a; Van Kooten Kok-Doorschodt et al, 1972) but these have been found to be either variations in the nomenclature of the aforementioned determinants or non-specific antigenic markers.

These determinants have significant epidemiological value. Not only do they tend to have a specific geographic distribution but are also more frequently associated with certain forms of the infection. The "d" determinant is found in asymptomatic carriers in North America, northern Europe, Asia and Oceania although "y" occurs in these areas as well. The "y" determinant on the other hand, occurs almost to the exclusion of "d" in Africa and is the more

common subtype in India and the Mediterranean area (Mazzur et al, 1974).

Our study confirms earlier reports that HB_sAg/ad is more commonly found in asymptomatic blood donors, renal dialysis patients and institutionalized populations while the ay subtype is predominant in acute disease (Schmidt et al, 1972; Holland et al, 1972, Iwarson et al, 1973; Perry and Chaudhary, 1973; Perry et al, 1975; Madden et al, 1975). The ad/ay ratio in our donor population was lower than that noted by other investigators (Holland et al, 1972; Dodd et al, 1973) but this may have been due to regional variations in antigenic subtypes which are known to occur (Dodd et al, 1973). A second consideration for this difference is the continuously ongoing shift in HB_sAg subtypes that has been occurring in the past 10-20 years. Magnus et al (1973) subtyped sera collected in 1953 from patients with acute hepatitis and found that they were predominantly HB_sAg/ad; by the late 1960's and early 1970's, HB_sAg positive sera from a similar population were largely ay.

Institutionalized populations tend to be closed, adynamic units and newly infected individuals tend to have the subtype common to that centre. Occasionally, a patient with a different HB_sAg subtype is admitted and pockets of this new antigen subtype are isolated (Madden et al, 1975).

Further HB_sAg characterization was possible only after antigen purification. We tried many of the purification schemes reported in the literature but found them to be either laborious and time consuming or technically

difficult to do. The two procedures presented in this study are modifications of previously described techniques (Gerin et al, 1969; Gerin et al, 1971; Gerin et al, 1975). These proved to be relatively simple to perform in comparison with other methods of HB_sAg purification and were consistently reproducible. The advantages of the large volume procedure are readily apparent - large volumes of serum can be purified in a minimal amount of time. The disadvantages include the need for more expensive centrifugation equipment, greater technical expertise on the part of the operator and the need for large volumes of costly CsCl. The small volume procedure, on the other hand, can be carried out using less expensive machinery, and smaller volumes of CsCl; however, two additional centrifugations add not only time to this procedure but also decrease effective HB_sAg recovery, albeit only in the range of 5-10%.

HB_sAg recovery by the two techniques was between 50 and 70% of the estimated antigen content in the starting sample. Precipitation of this antigen from serum by PEG 6000 increased recovery to approximately 90% and this simple procedure appeared to offer an ideal method for obtaining maximal antigen recovery; unfortunately, we were unable to remove all of the PEG 6000 from our final purified preparations despite repeated rate separation centrifugations and we had to abandon this promising technique as unworkable.

The final purified product was a homogeneous population of 22 nm spherical particles with a buoyant density of approximately 1.20 g/cm³ in CsCl. There were no

differences in size between the two subtypes. Specific antibodies directed against HB_sAg were raised by injecting the purified antigen into guinea pigs. Occasionally, antibodies to human serum albumin (HSA) were also found, even though no HSA was detectable in our final purified HB_sAg preparations. There had been some early reports that human host components were incorporated into the viral genome (Burrell, 1975); more recent evidence tends to confirm this and specifically shows this to be HSA. Imai et al (1979) have shown that HB_sAg from HB_eAg-positive individuals contain receptors for polymerized HSA and that red blood cells coated with polymerized HSA can be agglutinated by these particles. Hollinger and Dreesman (1979) reviewed this paper and speculated that both the HB_sAg and albumin antigenic determinants might reside on the same polypeptide. Subsequent investigations have found this not to be the case. Shih et al (1980) fractionated HB_sAg on polyacrylamide gel and found that between 20 and 40% of HB_sAg particles contained a P6 fraction which was shown to be HSA. This fraction could be precipitated with anti-HSA; however, coprecipitation of P6 with anti-HB_s and anti-HSA led them to conclude that the HB_sAg and HSA determinants resided on separate molecules. Other investigators reported that HB_sAg activity resided on a 49,000 molecular weight polypeptide and that this fraction expressed the identical subtype determinants and antigenic titres as intact HB_sAg. In addition, this polypeptide was as effective as HB_sAg in raising specific antibodies (Mishiro et al, 1980).

The NIH hepatitis B vaccines presently undergoing clinical trials were prepared in a manner similar to that described for the large volume purification procedure. The final purified product was formalin inactivated and 0.1% HSA was added to maintain HB_sAg structural integrity (Gerin, personal communication). The fact that this antigen was prepared from a human serum source and that human host components were part of the antigen composition has caused some investigators to worry about inducing autoimmune disease in vaccinees (Zickerman, 1976). Recently, investigators have been able to combine hepatitis B specific DNA with a plasmid and introduce this product into Escherichia coli with the resultant expression of hepatitis B specific DNA and protein by bacteria (Burrell et al, 1979; Valenzuela et al, 1979; Charnay et al, 1979). Cloning of the hepatitis B genome in bacterial cells holds much promise as an alternate method for producing viral vaccines both safely and efficiently.

Two distinct populations of Dane particles were identified in the sera of some HB_sAg-positive individuals based on electron microscopic observation of their staining patterns and on their different densities in CsCl. Particles were designated as either "empty" or "full" depending on whether or not they were penetrated by stain; empty and full Dane particles banded at 1.20 g/cm³ and 1.25 g/cm³ respectively. Our findings were in agreement with an earlier report by Gerin (1974) but differed slightly from a subsequent study which found these particles to band at 1.20 g/cm³ and 1.22 g/cm³ (Kaplan et al, 1976). In addition, we found

DNA polymerase activity to be associated with the denser Dane particle population and our findings were consistent with those of other investigators (Moritsugu et al, 1975; Kaplan et al, 1976; Macaya et al, 1979).

Lipman et al (1973) isolated cores from Dane particles by disrupting these particles with NP 40 and centrifuging them in a Ficoll-sucrose density gradient. Two distinct populations of cores were identified by electron microscopy; cores banding at a density of 1.29 to 1.30 g/cm³ in CsCl had smooth surfaces and reacted with anti-HB_c whereas cores banding at a density of 1.28 to 1.29 g/cm³ had a ragged surface and did not react with anti-HB_c. It was postulated that the ragged border of the non-reactive core preparation consisted of a "matrix protein" with a different antigenic specificity than the core. Moritsugu et al (1975) found that these "matrix proteins" were either anti-HB_c attached to the isolated particles as a result of the purification procedure or incompletely removed HB_s Ag.

We were also able to isolate two distinct populations of core particles from Dane particle preparations. The densities of the "empty" and "full" core particle preparations were 1.30 g/cm³ and 1.36 g/cm³ respectively with DNA polymerase activity associated with the denser particle. Our findings confirmed an earlier report by Kaplan et al (1976) who also described two subpopulations of cores with similar DNA polymerase activity in the denser population. Moritsugu et al (1975) also isolated two populations of

cores with densities of 1.30 and 1.36 g/cm³ but found DNA polymerase activity in both populations. They also found that the lighter core population had human immunoglobulin, presumably anti-HB_c associated with it and the density of these lighter particles was dependent on the degree of antigen-antibody association. Furthermore, the lower density core population was more heterogeneous in that it contained both higher density DNA polymerase-positive cores and lower density DNA polymerase-negative particles and as a result, the overall enzymatic activity of this population was lower than the higher density core preparation.

Our inability to detect DNA polymerase activity in the lower density Dane and core particle preparations may be due to a number of factors. The enzyme may not be present in the cores of these particles. A second possibility is that the enzyme is present but that the particles do not contain a template DNA to produce detectable DNA polymerase activity. A third consideration is that these Dane particles lack both the enzyme and the nucleic acid template. Kaplan et al (1976) feel that these Dane particles lack the appropriate nucleic acid template. The enzyme is needed for viral replication and it would seem highly unlikely that it would be lacking; in any case, such deletions would be strongly selected against. With no template, the particles would have no demonstrable DNA polymerase activity, regardless of whether the enzyme were present or not. The existence of such particles is not unique to hepatitis B since most virus preparations also contain variable numbers of "empty"

particles (Parks et al, 1967; Jacobson and Baltimore, 1968; Grady et al, 1970).

At present, no sera have been found to have DNA polymerase inhibiting activity and therefore, the DNA polymerase assay is not affected when utilized to quantitate DNA-polymerase positive Dane particle preparations (Hess et al, 1980). The DNA polymerase assay however is not a routine diagnostic procedure at present because the test system is variable and results do not add substantially to patient care and evaluation. Recently, it has been shown that serial determinations of DNA polymerase activity are the most sensitive indicators of a therapeutic effect during therapy of chronic hepatitis with interferon and other drugs and the use of this assay has strongly been advocated to monitor the efficacy of the various treatments (Merigan et al, 1980; Deinhardt, 1980).

Recently, sensitive assays have been developed to detect anti-HB_c of the IgM class which is a highly sensitive indicator of recent infection and clearance of the hepatitis B virus (Cohen, 1978; Katchaki et al, 1979; Gerlich and Luer, 1979). Anti-HB_c IgM is the first antibody to arise and it appears in or shortly before the acute stage of hepatitis B. It persists for several months but recent evidence suggests that it may be detectable for up to 17 months from the onset of illness (Hawkes et al, 1980). It is extremely rare for IgM antibodies to persist for more than 1-2 months in viral infections but there have been reports of prolonged IgM antibody responses of up to 4 months

in occasional rubella infections (Robertson and Bell, 1974). Disappearance of anti-HB_c IgM may prove to be the most reliable indicator for clearance of the hepatitis B virus whereas persistence denotes chronic disease and viral replication. High titres of this antibody are encountered in acute and convalescent phases of the disease while chronic active and persistent hepatitis have lower anti-HB_c IgM titres. The height of the titre does not correlate well with the severity of the disease and other markers should be used to determine this feature of the illness (Deinhardt, 1980).

Core particles were also isolated from liver nuclei of a chronically infected renal dialysis patient at necropsy. Particles banded at 1.30 g/cm³ in CsCl and were negative for endogenous DNA polymerase activity. Hirschman et al (1974) also isolated naked core particles from livers of chronic hepatitis B patients and showed DNA polymerase activity was present only in the presence of exogenous DNA template. Similar results were obtained from the necropsied liver of a chimpanzee with chronic active hepatitis (Bradley et al, 1976). Other investigators, however, have demonstrated the existence of endogenous DNA polymerase activity in hepatic tissue from humans and chimpanzees (Fields et al, 1976; Onda et al, 1978). The reason for these differences is speculative. Gerin et al (1975a) have suggested that the bulk of Dane particles, which appear to be "empty" and therefore deficient in nucleic acid, could play a role in the persistence of hepatitis B virus by acting as defective interfering particles. Livers obtained from chronically infected humans and animals may have contained many defective

core particles with DNA polymerase activity but no endogenous DNA template. HB_cAg particles isolated from humans and chimpanzees with little or no anti-HB_c in their plasma may on the other hand have a greater proportion of intact versus defective particles and may have the necessary template to initiate an appropriate DNA polymerase response (Onda et al, 1978).

The close association of DNA polymerase with HB_eAg led some investigators to speculate that HB_eAg may be the DNA polymerase protein (Melnick et al, 1976). Neurath and Strick (1976) showed that HB_eAg and DNA polymerase were not identical and in a later study, demonstrated that HB_eAg is a dimer of a 7S immunoglobulin (Neurath and Strick, 1977). They concluded that since HB_eAg and anti-HB_e are both immunoglobulins which react only with each other and not with normal IgG, the HB_eAg/anti-HB_e system represents an antibody/anti-antibody system. Subsequent studies have, however, challenged their conclusions (Takahashi et al, 1978).

Neurath et al (1976) also reported that HB_eAg was exposed on the surface of Dane particles and tubular forms of HB_sAg but were not present on the surface of the 20 nm spherical particles. Gerin et al (1978), however, were unable to find HB_eAg on the surfaces of Dane particles, filaments and 20 nm HB_sAg forms. Significantly, HB_eAg was not found on the surface of the population of Dane particles that contains DNA polymerase and correlates with hepatitis B infectivity indicating that anti-HB_e has no direct role in virus neutralization. Earlier, Lam et al (1977) reported

that treatment of Dane particle-rich preparations with Tween 80 released HB_eAg suggesting that HB_eAg represents an internal structural protein of the HBV. Their results were somewhat tainted by the finding that their preparations contained anti-HB_c and that this antibody may have resulted in an erroneous interpretation of their data (Huang, personal communication). However, subsequent studies tend to support an association between HB_cAg and HB_eAg. Mild detergent treatment of Dane particle-rich preparations resulted in the release of core particles containing HB_eAg activity when examined by reverse passive hemagglutination. When exposed to stronger detergent, additional HB_eAg activity was released only from intact core particles with DNA polymerase activity but not from empty core particles (Ohori et al, 1979). Takahashi et al (1979) isolated HB_cAg from Dane particles by treatment with NP 40 and β-mercaptoethanol. HB_eAg was not detected on the surface of the Dane particles or the resultant cores. Treatment of the core with pronase resulted in some HB_eAg activity while further treatment with SDS yielded high HB_eAg activity, indicating that HB_eAg exists in the core of the Dane particle in a cryptic form. They concluded that HB_eAg is an integral component of the Dane particle and accounts for the close correlation between HB_eAg and Dane particles in HB_sAg positive sera. HB_cAg purified from hepatic nuclei was converted into HB_eAg by sonication or by passing it through an anti-HB_c IgG-conjugation sepharose B column; however, there was no appreciable difference in protein composition between HB_cAg

and HB_eAg. Centrifugation in CsCl also converted core particles to HB_eAg. Ohori et al (1980) hypothesized that HB_cAg resides on a protein conformation which consists of a polypeptide sharing HB_e antigenicity.

HB_eAg has been proposed as a marker of infectivity by many investigators. Okada et al (1976) showed that in a group of mother-child pairs, all infants born to HB_sAg and HB_eAg-positive mothers developed HB_s antigenemia; conversely, all HB_sAg and anti-HB_e-positive mothers had infants who did not develop HB_sAg. Beasley et al (1977) also found maternal e antigenemia to be a good predictor of vertical transmission. In addition, studies of accidentally inoculated medical personnel also showed a striking correlation between HB_eAg and infectivity (Grady, 1976; Alter et al, 1976). Other investigators, however, have found that anti-HB_e positive sera may also be infective. Berquist et al (1976) inoculated four chimpanzees with HB_sAg positive sera containing anti-HB_e. They found that a human serum with anti-HB_e was infectious for one chimpanzee and that an anti-HB_e-positive chimpanzee serum may have been infectious for a second animal. Subsequently, investigators have also been able to detect HB_sAg infants born to HB_sAg, anti-HB_e-positive mothers; in all three studies, antigenemia was transient and the infants later developed anti-HB_s (Schweitzer et al, 1975; Gerety, 1976; Gerety and Schweitzer, 1977). Gerin et al (1978) found that anti-HB_e had no direct role in virus neutralization and that the correlation between anti-HB_e and lack of infectivity was probably an indirect one. This

finding may serve to explain the equivocal data presented.

The prevalence of HB_eAg in the four populations we studied was comparable to or higher than that reported in other studies of asymptomatic blood donors and other healthy HB_sAg carriers (Magnius and Espmark, 1972b; Magnius et al, 1975; Nordenfelt and Kjellen, 1975; El Sheikh et al, 1975; Feinman et al, 1975; Smith et al, 1976; Takahashi et al, 1976; Skinhøj, 1977; Couroucé-Pauty and Plançon, 1978) but lower than that reported by others (Hindman, et al, 1976; Maynard et al, 1976; Werner et al, 1977). We also found several HB_sAg-positive sera which were negative for HB_eAg but positive for DNA polymerase and vice versa. Similar results were obtained by other investigators (Imai et al, 1976). A 44% correlation was found in our study between HB_eAg and DNA polymerase activity which was comparable to findings by some researchers (Cappel et al, 1978) and considerably higher than results in other studies (Imai et al, 1976). Concentration of HB_eAg-positive sera further increased the correlation of HB_eAg and DNA polymerase activity to 80%.

The discrepancies between results can be attributed to three factors: the insensitivity of the assay used, the pre-selection of sera for testing by some investigators and the probable regional variations that exist similar to those reported for HB_sAg. All aforementioned studies used the relatively insensitive immunodiffusion technique. The RIA increased sensitivity by up to tenfold (Mushawar et al,

1978; Frösner et al, 1978) and it is reasonable to assume that many of the sera negative for HB_eAg by AGD and positive for DNA polymerase will, in reality prove to be HB_eAg-positive by more sensitive assays. A second consideration is sera pre-selection. Werner et al (1977) selected high-titred HB_sAg-positive sera and found that more than 50% of all sera tested were HB_eAg-positive. An association has been found between the presence of HB_eAg and high titres of HB_sAg in sera (Okada et al, 1976; Imai et al, 1976; Trepo et al, 1976; Beasley et al, 1977; Skinhøj, 1977) and this may account for the large number of HB_eAg-positive specimens in their study. This can also account for the increased frequency of HB_eAg-positive sera in dialysis patients who generally tend to have higher titres of HB_sAg.

Some investigators report that anti-HB_e indicates the absence of Dane particles (Imai et al, 1976; Hindman et al, 1976). We and others (El Sheikh et al, 1975; Werner et al, 1977) however, find Dane particles in certain sera positive for anti-HB_e. Werner et al (1977) extracted DNA from sera and assayed its ability to anneal to a [³²P]-DNA probe that was a copy of the Dane particle DNA. All 10 HB_eAg-positive samples tested contained DNA that formed specific hybrids with the DNA probe. One anti-HB_e positive sample also annealed with the radiolabeled DNA probe although the level of DNA hybridization was much lower than that of the HB_eAg-positive samples. Electron microscopic examination of this sample showed that it contained Dane particles. The Dane particles seen in our anti-HB_e positive samples

were all found to be negative for DNA polymerase activity and were empty when visualized by electron microscopy.

Alberti et al (1978) also found Dane particles from anti-HB_e positive sera to be empty.

In our study, we found that HB_sAg/ay positive sera from asymptomatic blood donors had a higher frequency of HB_eAg than did HB_sAg/ad. The anti-HB_e frequency was reciprocal for the two subtypes. Couroucé-Pauty and Plancon (1978) have found that the frequency of both HB_eAg and anti-HB_e is higher for HB_sAg/ad. The only group in which we found HB_eAg frequency to be higher for the ad subtype was the dialysis population. One can only speculate as to the reason for these significant differences. It has been noted previously that there has been a recent shift in the proportion of the two subtypes in the donor population. It appears that the HB_sAg/ay positive donors have been more recently infected than the chronic "ad" carriers; in addition, the HB_sAg/ay positive donors tend to be somewhat younger. Sasaki et al (1979) have recently shown that the frequency of HB_eAg decreases with both age and length of carriage while antibody frequency increases. This could explain our findings in the donor population. Dialysis patients, on the other hand, tend to have high titres of HB_sAg and HB_eAg prevalence has been found to be increased with increased titres of HB_sAg (Imai et al, 1976; Beasley et al, 1977; Sasaki et al, 1979).

RIA is certainly the technique of choice now in

determining HB_eAg prevalence; however, what it accomplishes in sensitivity it lacks in specificity in that it cannot differentiate between HB_e antigenic reactivities. Initially, Williams and Le Bouvier (1976) described two e determinants which they designated as e₁ and e₂. We, along with other investigators (Couroucé-Pauty and Plancon, 1978; Murphy et al, 1978) have found a third determinant which has been designated as e₃. This third line appeared in conjunction with e₂, disappeared at the same time and had the same biophysical characteristics. It seems from our studies that e₂ and e₃ are closely related but that they differ markedly from e₁. Therefore, it may be more appropriate to designate e₃ as e_{2a} to denote these similarities.

The significance of the e determinants is not known. Williams and Le Bouvier (1976) have speculated that the appearance of e₂ heralds HB_sAg clearance in the near future. This seems unlikely as many sera from chronic HB_sAg carriers contain both determinants and have for lengthy periods of time. A possible correlation however seems to exist between these determinants; DNA polymerase activity and Dane particle numbers. In patients with acute hepatitis B, DNA polymerase activity reached its peak when e₂ was detected in addition to e₁, and declined thereafter. Chronic HB_sAg carriers positive for both e₁ and e₂ had the highest numbers of Dane particles in their plasma when examined by EM. Our findings and suspicions were recently confirmed when Yamada et al (1979) found that although e₁ was more common than e₂, e₂

positive sera contained increased concentrations of Dane particles and had higher levels of DNA polymerase activity.

We also studied the prevalence of HB_eAg and anti-HB_e in two institutions for the mentally retarded and our findings confirm earlier reports that no significant differences exist between DS and OMR populations (Hindman et al, 1976). The number of HB_eAg positive sera was lower than in the aforementioned study but anti-HB_e was more prevalent. Recently, Gust et al (1978) have reported that significant differences existed between these two groups; their percentage of HB_eAg-positive sera was much higher than in our study while anti-HB_e was almost non-existent in their populations, a finding they did not attempt to interpret. They also found that HB_eAg prevalence was highest in the young, declining in the second and subsequent decades. Their findings echoed an earlier study by Ohbayashi et al (1976) who also noted a decreasing frequency of HB_eAg with age but in a non-institutionalized population.

Our experience has been somewhat different. The prevalence of HB_eAg and anti-HB_e was found to be more a function of length of residence rather than age at admission. Individuals in both the first and second decades had equal opportunity of becoming HB_eAg positive and we found that in our two centres, no significant differences existed between the two age groups. However, when length of residence was considered, significant differences were noted for both the DS and OMR populations; the only discrepancy

occurred with the DS group at the Huronia Centre. A significant number of DS patients had been admitted in the past ten years when compared with the Rideau Centre and a significantly smaller number of residents had become HB_eAg positive. It is difficult to explain why 67% of one institution had become HB_eAg positive whereas only 25% of another were positive. The small numbers studied make these differences insignificant; nevertheless, one might assume that these two institutions handle their new admissions somewhat differently, thereby accounting for these apparent discrepancies.

What is the significance of HB_eAg in determining the course of an acute hepatitis B infection? Aldershvile et al (1980) followed HB_eAg and anti-HB_e by RIA and found the course of events to be similar for the two patients we have described in our study. Fifteen of sixteen individuals infected with HB_sAg became HB_eAg positive two weeks after the onset of symptoms; antigen prevalence declined during the next ten weeks. Chronic liver disease was noted in five patients in whom HB_eAg persisted for more than ten weeks. The vast majority of patients became anti-HB_e positive (91%) with 82% having detectable anti-HB_e activity within two weeks of HB_eAg clearance. From these findings, they concluded that HB_eAg occurs regularly in early acute hepatitis B and that persistence may correlate with either a chronic carrier state or chronic liver disease. In another study, however, some question was raised about its significance. Schulman et al (1980) studied 71 susceptible

oncology patients who inadvertently received a tumor cell vaccine contaminated with HB_sAg; 45 became infected. Of these, 28 were positive for both HB_sAg and HB_eAg, 8 were positive for only HB_sAg, and 9 had detectable anti-HB_s. No significant difference was seen in the acute course and outcome between the two HB_sAg groups. They concluded that even though HB_eAg was common early in the course of the disease, it had no prognostic significance independent of HB_sAg.

The mechanism by which HB_eAg is cleared from the serum is poorly understood. Recently, Tabor et al (1980) detected HB_eAg by AGD in four patients with tropical splenomegaly syndrome; all four were negative for HB_sAg by RIA. Two patients subsequently developed anti-HB_s while the other two became anti-HB_c positive. Detection of HB_eAg by an insensitive assay would seem to indicate that removal of the two antigens occurs independently. These results, however, remain to be confirmed.

Much work has been done in attempting to correlate HB_eAg to HBV and in determining its prognostic value with regards to resolution of acute illness. Its association with a non-hepatic disease also raises questions about its specificity. Further studies are apparently needed in order to resolve these discrepancies and to ascertain the true significance of this marker in hepatitis B.

II. Cell Mediated Immunity

The cell-mediated immune response is a function of the T-lymphocyte and is important in the resolution of intracellular infections by viruses, fungi and mycobacteria and for tumor immunity and transplantation rejection. The T-cell population is not a homogeneous one but rather is composed of subsets of T cells consisting of mediator (lymphokine) producing cells, cytotoxic or killer (K) cells, memory cells, helper cells and suppressor cells. The latter two cell populations are differentiated from each other by their ability to bind with specific immunoglobulins. The cells with receptors for the Fc portion of IgM are the helper cells and make up approximately 70% of the total peripheral blood T cell population. They are designated as T_H or T_M cells. Cells with receptors for the Fc portion of IgG are the suppressor cells and are called T_S or T_G cells. Helper and suppressor activity also reside in the non-specific monocyte population. Monocytes may exert a suppressor effect on lymphocyte responses to mitogens, for example, by producing the prostaglandin PGE_2 which results in increased intracellular cyclic adenosine monophosphate (c-AMP) and decreased cellular function.

Four distinct lymphocytic cell populations are found in the peripheral blood. T-lymphocytes make up approximately 70% of peripheral circulating agranular white cells. They form spontaneous E-rosettes with sheep red blood cells, have no detectable surface immunoglobulins and have receptors

for the Fc portions of IgM and IgG. B lymphocytes make up about 10% of the peripheral monocytes. They also form rosettes but with mouse red blood cells. Unlike the T-lymphocytes, they have detectable surface immunoglobulin and have receptors only for the Fc portion of IgG. Monocytes comprise an additional 20% of this population; they do not form rosettes spontaneously, and have no surface immunoglobulin. They do, however, have receptors for the Fc portion of IgG. The fourth component of this population are the null cells which make up 0 to 5% of the agranular white cells in the peripheral blood. They do not form rosettes, have no surface immunoglobulin and may or may not have Fc receptors on their surfaces. It is thought that interactions between these four cell types are responsible for the in vivo and in vitro manifestations of cell-mediated immunity.

The cell-mediated immune response to hepatitis B has been measured by in vivo and in vitro techniques in animals and man. Delayed skin reactions to HB_SAg have been demonstrated in guinea pigs (Irwin et al, 1972), mice (Roberts et al, 1975) and chimpanzees (Ibrahim et al, 1974; Trepo et al, 1975). Gerety et al (1974) have found this reaction to be specific for the particular hepatitis B antigen used to sensitize the animal. Guinea pigs immunized with HB_SAg had a positive reaction only when skin tested with this antigen; HB_CAg-sensitized animals only responded to an HB_CAg challenge. No skin testing however has been done on humans because of the very real danger of inducing a hepatitis B infection. It, therefore, remains to be

proven whether the delayed hypersensitivity reactions observed in animals is truly representative of the human response.

The specific cell-mediated immune response to HB_sAg has also been shown to be transferrable to non-sensitized animals. Vyas et al (1974) phenol-extracted RNA from guinea pig lymph nodes immunized with HB_sAg and added it to peritoneal exudate cells from normal non-sensitized guinea pigs. In the presence of HB_sAg, migration by these peritoneal macrophages was inhibited. This transfer of specific cellular immunity by RNA is not unique to hepatitis B. Han (1973) had demonstrated the transfer of delayed skin reactivity to tuberculin, varidase and candida by injecting RNA from sensitized animals into non-sensitized animals.

In the present study, we have measured two in vitro parameters of the cell-mediated immune response - lymphocyte transformation and leukocyte migration. We also examined various other factors which might affect the ability of lymphocytes to respond in culture, namely the age of the cell donor, and the serum supplements used. Cells from older individuals were found to have a decreased ability to respond to a challenge by a powerful non-specific lymphocyte stimulant (PHA) as shown by a decrease in tritiated thymidine uptake than did cells from younger individuals. Our results were in agreement with earlier studies which noted that immunological responses decreased with increasing age (Price and Makinodan, 1972; Krogsrud and Perkins, 1977).

Recently, these findings have been expanded on by Rice et al (1979) who found three distinct suppressor systems in human blood that modulated lymphoproliferation. The first was an adherent cell suppressor system (ACSS) which consisted of steroid and radioresistant monocytic cells that survived well in culture. The second was designated the prostaglandin-related suppressor system or PgSS. These cells were similar to the ACSS cells morphologically but differed in their ability to suppress other cells. In addition, they had a lower effective concentration in the peripheral blood and were sensitive to indomethacin. The third cell in the suppressor triad was the ISS or induced suppressor system T-lymphocyte which was shown to be partially radiosensitive but unlike the two other cells, lost its activity in culture within 24 hours. ACSS and PgSS cells from older individuals were more active than cells from younger people and this may partially explain the age-related depression of the lymphocyte response to PHA found in our study. Age may also affect the receptors located on the responding cell surface either by decreasing the availability of sites or by decreasing their avidity for the stimulating agent and this too must be considered.

Non-specific stimulation of effector cells in CMI assays is always a concern. Fetal bovine serum which is a heterologous serum supplement when added to human lymphocyte or leukocyte cultures is known to induce non-specific stimulation of cells (Oppenheim and Schecter, 1976) and

probably should not be used. Autologous serum, on the other hand, often tends to be inhibitory and occasionally cytotoxic and may dampen CMI responses to specific antigens (Bloom et al, 1973). Most investigators use pooled AB negative serum in an attempt to minimize these effects; however, there is no evidence at present to indicate that ABO and Rh determinants are stimulatory or inhibitory and use of AB negative serum may be an unnecessary precaution. Antibiotics also play a role in the cellular response to stimulants in culture. Munster et al. (1977) found severe dose-dependent suppression of DNA synthesis in the presence of minocycline, oxytetracycline and the ascorbic acid salt of tetracycline. Less severe but still significant suppression was found in the presence of chloramphenicol, clindamycin, tetracycline and ascorbic acid alone. No effect was noted when penicillin, carbenicillin or cephalothin was added and slight stimulation was noted in the presence of gentamicin. Other investigators found that leukocytes incubated with chloramphenicol, rifampin, sodium fusidate and tetracycline had markedly depressed migration when compared to other antibiotics (Forsgren and Schmeling, 1977).

In the present study, cells from patients who had recovered from an acute HBV infection showed no significant lymphocyte transformation when incubated with purified HB_sAg, an observation diametrically opposed to earlier reports which found lymphocyte transformation to be detectable up to six years after recovery from an acute hepatitis B

infection (Yeung Laiwah, 1971; Pettigrew and Russell, 1972; Tsuji et al, 1972; Sodomann and Havemann, 1973; Yeung Laiwah et al, 1973; De Gast et al, 1973; Koszinowski et al, 1974; Reisenbuk and Ioks, 1974; Chandra, 1975; Tong et al, 1975). Koszinowski et al (1974) found that anti-HB_s was needed for lymphocyte transformation while De Gast et al (1973) reported that by heat-inactivating HB_s Ag, they could further enhance tritiated thymidine uptake by cells from post hepatitis B patients. Fourteen of our 16 patients had detectable anti-HB_s in their plasma but in none of them was the lymphocyte transformation response significant. We also tested two inactivated HB_s Ag preparations - heat-inactivated HB_s Ag (results not reported) and the formalin-inactivated NIH hepatitis B vaccine - and found no significant transformation with either.

More recent studies, however, have shown that lymphocytes from post hepatitis B patients incubated with highly purified HB_s Ag preparations do not undergo significant transformation (Wands et al, 1975; Tikku et al, 1978; Hafkin et al, 1979). Wands et al (1975) were also unable to demonstrate cytotoxicity with lymphocytes from this patient population and concluded that "factor(s) other than HB_s Ag are involved in the stimulation of recovered hepatic patient lymphocytes".

One such factor may be the presence of serum components or contaminants in the antigen preparations used; some of the earlier experiments were performed with either HB_s Ag-positive sera or partially purified HB_s Ag. We tested

four such serum contaminants obtained during the course of our purification procedure and found one to significantly stimulate lymphocytes from post hepatitis B patients; fractions from normal human serum processed in a similar manner had no effect. We did not attempt to determine what specifically was causing this stimulation but one and possibly more factors may be involved. The stimulant may be a fragment or possibly a major component of either the HBV or HB_sAg dissociated from these particles during the purification procedure; fraction 1 was weakly positive for HB_sAg by RIA but we were unable to visualize any fragments, 22 nm spherical particles, filaments or Dane particles by EM. A second possible candidate is some new or perhaps altered antigen released from the liver into the circulation during the disease process. Kissling and Speck (1971) examined 27 sera from patients with various liver diseases and found 25 of them caused normal lymphocytes to become blastoid and undergo mitosis. Since all sera had a comparable transforming effect on lymphocytes and since only two were positive for HB_sAg, it did not appear that this antigen had a direct effect on transformation. These findings and similar results by Barinsky et al (1975) suggested that perhaps hepatic antigens or products might be the stimulating agent. Tobias et al (1967) had found that autologous liver homogenates from patients with liver disease could transform lymphocytes in culture, expanding on earlier findings by Geduldig and Iber (1962) that some patients with different liver diseases had a delayed cutaneous hypersensitivity

response when challenged with autologous hepatic tissue suspensions. These results have now been confirmed by other investigators and have led some to speculate that the pathogenesis of chronic liver disease may be related to the induction of the immune response to host tissue proteins such as liver specific antigens (Bacon et al, 1971; Miller et al, 1972; Popper and Mackay, 1972; Smith et al, 1972; Knolle et al, 1973; Thomson et al, 1974; Edgington and Chisari, 1975; Cochrane et al, 1976; Hopf et al, 1976; Tiku et al, 1978).

No standardized HB_sAg preparation has been used to determine in vitro CMI responses because of the differences in the purification procedures that exist from laboratory to laboratory and may, in part, also account for the different results obtained by various investigators. Some investigators have been unable to reproduce their results when moving from one centre to another. Tiku et al (1978) reported significant lymphocyte transformation in 12 of 17 chronic HB_sAg carriers with elevated serum glutamic pyruvic transaminase and 1 of 2 patients with chronic hepatitis B. However, when Tiku tried to reproduce these results at another centre using identical techniques and antigen preparations and a similar patient population, he was unable to do so (Hafkin et al, 1979).

Differences in lymphocyte stimulation using highly purified versus semi- or unpurified antigens are not unique to hepatitis B. Investigators have been able to stimulate lymphocyte cultures with unpurified, measles-infected cell

membranes or measles vaccines (Graziano et al, 1975; Ruckdeschel et al, 1975; Cunningham-Rundles et al, 1975) while purified measles antigen was found to be inhibitory (Zweiman and Miller, 1974; Sullivan et al, 1975; Arstila et al, 1976; Ilonen, 1979).

We also measured the CMI responses of post-hepatitis B patients by the leukocyte migration inhibition assay previously described by Clausen (1971). This test was preferred to the capillary tube technique because it used less antigen and fewer cells, thus facilitating the simultaneous testing of several antigenic concentrations. The overwhelming majority of the migrating cells were found to be polymorphonuclear leukocytes and our findings are consistent with earlier findings which reported similar results. (Erard, 1974). When examining our population as a whole, no significant inhibition of cellular migration was found, confirming results reported by other researchers (Dudley et al, 1972b; Gerber et al, 1974; De Moura et al, 1975). However, two groups have reported inhibition during a period of several months of convalescence (Frei et al, 1973; Yeung Laiwah et al, 1973) while another noted the simultaneous occurrence of migration inhibition and anti-HB_s (Reed et al, 1974). These discrepancies may be due to the purity of the antigen used and/or to technical differences. Ito et al (1972) used liver homogenates from HB_s Ag-positive liver biopsies and demonstrated leukocyte migration inhibition in patients with a previous history of HB_s Ag.

When we examined our population on an individual basis, we noted that the two patients who had just cleared HB_s Ag within a two week period and were anti-HB_s negative had significant migration inhibition of their leukocytes when cultured with two concentrations of purified HB_s Ag. Ibrahim et al. (1975) also noted a similar pattern in their population and concluded that the disappearance of circulating HB_s Ag after acute hepatitis coinciding with the appearance of leukocyte migration inhibition and not with humoral immunity is consistent with the hypothesis and observations of Dudley et al (1972a) that the cellular immune response plays an important role in the clearance of this infective agent.

Why lymphocytes previously exposed to herpes simplex virus, rubella, varicella, mumps and cytomegalovirus respond to these specific viral antigens in vitro (Simons and Fitzgerald, 1968; Russell et al, 1972; Russell, 1974; Rasmussen et al, 1974; Vesikari and Buimovici-Klein, 1975; Starr et al, 1975; Møller-Larsen et al, 1976; Rossier et al, 1977a, b; ten Napel et al, 1977; Møller and Soren, 1977; Pollard et al, 1978; Arvin et al, 1978; Ilonen, 1979; Hafkin et al, 1979) while cells exposed to HB_s Ag do not is open to speculation. One possible explanation for this finding is that a population of suppressor T-cells somehow inhibits lymphocytes sensitized to HB_s Ag from recognizing and/or responding to a challenge by this antigen. Until recently, such T-T cell interactions had only been described for

animals, the classic model being the Ly system in mice (Cantor et al, 1978; Eardley et al, 1979a, b). Antigen stimulated Ly 1 cells were capable of inducing B cells to produce antibodies and non-immune T cell subsets (surface phenotype Ly 123⁺; Qa 1⁺) to participate in suppressor activity by feedback inhibition. The Ly 1 cell in this system is a helper cell and behaves like the T_M cell in man while the Ly 2 cell could act either as a suppressor (analogous to the T_G cell) or as a K-cell. In 1979, Lobo and Spencer demonstrated T-T cell interactions in man. One suppressor cell subset activated by concanavalin A (Con A) was found to inhibit a blastogenic response to allogeneic cells while a PHA-activated population inhibited the generation of K-cells. These differed from the T-suppressor subset involved in the suppression of plasma cells (T-B interaction) in that they were resistant to both radiation and prednisone. Their findings and those of Rice et al (1979) discussed earlier would indicate that the suppressor cell population is a heterogeneous one. Separation of the T-lymphocyte population into its various subsets and challenging each one with purified HB_sAg would certainly be one way of resolving this issue and would also give us a better understanding of the significance of the cell to cell interactions that occur during the disease process.

The inability of sensitized lymphocytes to respond to HB_sAg may be a manifestation of tolerance. Two theories exist. One is the clonal deletion model advocated by Burnet (1959) in which self-reactive lymphocytes are thought

to be eliminated upon contact with self antigens. The second is the clonal abortion theory which holds that as lymphocytes acquire immunological competence, there is a particular stage in the differentiation process during which contact with self-antigen results in the elimination of potential self-reactive clones (Nossal, 1958). Human host components, particularly HSA, have been shown to be incorporated into HB_SAg and may result in this antigen being recognized as self. HB_CAg, on the other hand, contains no human host components and strong CMI responses have been obtained using this antigen (Gerety et al, 1974); unfortunately, it is difficult to obtain large amounts of this antigen and this has hampered efforts to fully assess CMI responses to it. Recently, two developments have occurred which may offer ways in which to resolve these problems. Mishiro et al (1980) have isolated a 49,000 m.w. polypeptide containing all the determinants and immunogenicity of the intact HB_SAg particle but without HSA which is found to reside on another polypeptide (Shih et al, 1980). Stimulation with this antigenic subunit would certainly circumvent any inhibition by HSA (if it does indeed exist) and could possibly resolve the discrepancies that exist in the literature. The second development is the finding that the hepatoma cell line from which HB_SAg has been obtained also produces HBV specific DNA (Marion et al, 1980); cultures of this cell line may therefore be a potential source for the large amounts of HB_CAg needed to fully assess CMI responses to this antigen.

Much work has already been done in trying to understand the role of the cell-mediated immune response in the pathogenesis of liver disease but the results obtained to date raise as many questions as they answer. Despite the massive amount of research done in the past ten years, much more still remains to be done before we can fully appreciate the intricacies of the various cell populations involved in clearing this infection and the significance of each of the antigen markers detected.

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APPENDIX I

Nomenclature of Antigens Associated with
Viral Hepatitis Type B

by

Committee on Viral Hepatitis of the
National Research Council
National Academy of Sciences

- HB_S Ag The hepatitis B antigen found on the surface of the Dane particle and on the unattached 20 nm particles.
- HB_C Ag The hepatitis B antigen found within the core of the Dane particle.
- Dane particle A current term for the 42 nm particle containing the HB_C Ag in its core and HB_S Ag on its surface.
- HBV Reserved for hepatitis B virus. The Dane particle may turn out to be HBV.
- HB_S Ag/adr Hepatitis B surface antigen manifesting the group specific determinant, a, and subtype-specific determinants, d and r. All recognized

subtypes are to be indicated to the right of the slash.

anti-HB_s Antibody to hepatitis B surface antigen. If the subtypic reactivity is known, the appropriate antigenic determinants are to be indicated to the right of a slash.

anti-HB_c Antibody to hepatitis B core antigen. If more than one core antigen is discovered, the corresponding antigens can be indicated.

Source: Morbidity and Mortality Weekly Report 23:29, 1974.

CONTRIBUTION TO KNOWLEDGE

The investigations performed in this study make the following original contributions to hepatitis B research:

- (1) Contrary to earlier reports, the CMI response of convalescing hepatitis B patients is in part dependent on the purity of the antigen employed. In our hands, no purified HB_sAg preparations (including the HB_sAg vaccine) were able to stimulate lymphocyte transformation. Furthermore, no significant migration inhibition could be demonstrated in this population using four different concentrations of highly purified HB_sAg.
- (2) Contaminating serum components recovered during the purification procedure were shown to significantly stimulate lymphocytes from convalescent HB_sAg patients. No attempt was made to specifically identify these stimulatory substances but it seems likely that their inclusion with partially purified antigen preparations in earlier experiments led to erroneous conclusions by these investigators on the role of CMI in hepatitis B.
- (3) A small volume purification procedure was developed. This had the advantages of being rather simple to perform, cost effective and consistently reproducible.
- (4) Further epidemiological data on the e antigen and antibody is provided. HB_eAg was more frequently

found to be associated with HB_sAg/ay than HB_sAg/ad whereas anti-HB_e was more frequently associated with the latter subtype. This may, in part, explain the recent shift from the more common ad subtype in North America to HB_sAg/ay in both acute and chronic hepatitis B infection.

- (5) A third e antigen marker is described. Because of its similarities to e₂ (time of appearance and disappearance, biophysical properties), it has been designated e_{2a}.