EXPERIMENTAL VIROLOGY

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HEPATITIS
VIRUSES
OF MAN

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PREFACE

The last decade has seen rapid and indeed dramatic progress in the identification of viruses causing hepatitis in humans, starting in 1968 with the discovery of the association between Australia antigen and hepatitis type B. Five years later hepatitis A virus was identified in faecal extracts, and more recently evidence has been obtained of a third form of hepatitis, which may be caused by more than one virus. The unprecedented effort which has been devoted to the problem of viral hepatitis in many countries of the world by epidemiologists, virologists, molecular biologists, pathologists, immunologists, clinicians, blood transfusion services and public health authorities, to name but a few disciplines, reflects the impact of these viruses on human health and welfare.

Rapid advances during the last five years have also resulted in the development of highly unusual vaccines against hepatitis B; the identification and characterization of hepatitis A virus and a preliminary report on the cultivation of this virus in tissue culture; and recently published reports on the insertion of hepatitis B virus DNA fragments into *Escherichia coli* plasmid with subsequent production of clones.

This monograph, however, is not an encyclopaedic review of viral hepatitis, which was first documented in the fifth century B.C., but an account of the more important published advances and exciting developments in this field. In general, key references are listed from 1975 onwards. Earlier books on the subject (*Virus Diseases of the Liver*, 1970; *Hepatitis-Associated Antigen and Viruses*, 1972 and *Human Viral Hepatitis*, 1975) carry extensive references to the world literature up to 1975.

It is a particular pleasure to acknowledge with thanks the hard work and enthusiasm of members of the staff of the Hepatitis Research Unit of the Department of Medical Microbiology, my co-author Dr C. R. Howard, Dr K. N. Tsiquaye, Dr Jacinta Skelly, Mr G. F. Mann, Mrs Anthea Thornton, Mrs Hazel Smith, Miss Jill Dixon, Miss Caroline Stanley, Mrs Jenny Copeland, Miss Ruth Smith, Mr M. Bowerman; my visiting Research Fellows, Dr Tzenny Kremastinou-Kourea (University of Athens) and Dr Yehudit Elkana (The Hebrew University-Hadassah Medical School) and my colleague Dr R. G. Bird.

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1 | The history of viral hepatitis

THE ORIGIN OF THE TERM JAUNDICE

The word jaundice is derived from the French *jaune*, yellow. The technical term *icterus* is less appropriate, being the Greek word for the golden thrush, a bird with golden plumage, the sight of which by a jaundiced person was believed by the ancients to be death to the bird, but recovery to the patient. Aretaeus, the Cappadocian (A.D. 81–138) gave a different derivation for jaundice:

> For it is a dire affection, the colour being frightful in appearance, and the patients of a golden colour; for the same thing is not becoming in a man which is beautiful in a stone. It is superfluous in me to tell whence the name is derived from certain four-footed and terrestrial animals, called ικτιδες (a species of ferret, either the *Mustela erminea* or the *M. furo*), whose eyes are of this colour.
>
> If a distribution of bile, either yellow, or like the yolk of an egg, or like saffron, or of a dark-green colour, take place from the viscus, over the whole system, the affection is called Icterus, a dangerous complaint in acute

diseases, for not only when it appears before the seventh day does it prove fatal, but even after the seventh day it has proved fatal on innumerable instances.

There are two species of the affection; for the colour of the whitish-green species either turns to yellow and saffron, or to livid and black.

In the whiter species, the patients are of a light-green colour, and more cheerful in mind; slow in beginning to take food, but eat spiritedly when begun; of freer digestion than those of the former species; alvine discharges, white, dry clayey; urine bright-yellow, pale, like saffron.

It is familiar to adolescents and young men, and to them it is less dangerous; it is also not altogether unusual with children, but in them it is not entirely free from danger.

EPIDEMIC JAUNDICE UNTIL THE NINETEENTH CENTURY

Epidemic jaundice was known to Greek and Roman writers in the fifth century B.C. and it is mentioned in the *De internis affectionibus*, as the fourth kind of jaundice. In the Epidemics, Books I and III, jaundice in epidemic form is mentioned, but exact interpretation is difficult since yellow bile was regarded as one of the four humours and the agent held responsible for most fevers. The description could have been that of relapsing fever or of malaria. Frerichs (1861) pointed out that the ancient physicians designated certain groups of functional derangements such as inflammation of the liver, "with an anatomical origin of which they were but imperfectly acquainted". Hence an unclear idea was attached to the term. Galen (A.D. 130–200) distinguished between phlegmon and erysipelas of the liver, and, in addition to inflammation, described a cold and a hot "intemperies". Bianchi designated this intemperies by the term *hepatitis* (Hepatitis est inflammatio hepatis non exquisite legitima). He mentions three varieties, hepatitis calida, frigida and mixta, and made phlegmon and erysipelas of the liver distinct from it.

It seems that the contagious nature of jaundice was first implied in the eighth century A.D. in letters from Pope Zacharias to St Boniface, Archbishop of Mainz:

. . . You ask what should be done about horses or men who are suffering from jaundice. In the case of men, if they have been ill thus from birth, they ought to dwell outside the city and avoid accepting alms from the people. If, however, they were not infected at birth, but are afflicted by a suddenly appearing illness, they should not be cast out, but should be cured

if possible. . . . Horses which have previously been infected with this
disease, if they cannot be cured, should be cast out into the pits, lest their
illness infects the others by contact (Migne, 1863).

Accounts of epidemics of jaundice among civilians appear from July and
August, 1745. The earliest report was from Minorca (Cleghorn, 1776,
1779). This was followed by a report from Brasov, Romania in 1784–1785
(Lange, 1791), and reference was made to Brunning's book on epidemic
spasmodic jaundice in Essen in 1772. A large epidemic of jaundice
occurred on the Ligurian coast between January and March, 1973 and an
epidemic which ran a mild course took place in Ludenscheid in 1794.

CATARRHAL JAUNDICE

Bonnet (1828–1841) was the first to attempt to show that almost all the
forms of disease of the liver arose from inflammation of that organ, and
Richard Bright (1836) considered inflammation of the liver as one of the
four causes of jaundice:

> Inflammatory action of the liver, which chiefly gives rise to jaundice, occurs
> in its substance; sometimes if unchecked going on to suppuration; but at
> other times producing a gradual change in the texture of the liver.

The view that infective jaundice was obstructive and not hepatic in
origin was first suggested by Bamberger in 1855, who considered that
swelling of the ostium of the common bile duct was the principal cause
of jaundice. This view, however, is generally ascribed to Virchow, who
described in 1865 the pathology of hepatitis after the examination of a
single case in which the terminal portion of the common bile duct was
plugged by mucus from the duodenum. The onset of the disease was always
associated with a gastro-intestinal upset and it was assumed that a micro-
bial infection, not necessarily specific, spread upwards from the intestine
to block the bile duct by catarrhal inflammation or cholangitis and thus
the term "catarrhal jaundice" was introduced. Fröhlich, when reviewing
30 outbreaks of jaundice in 1879, reported that there was a suggestion
that an infectious process might be implicated in only one outbreak. This
fitted well with the then widely accepted view that all types of jaundice
were essentially obstructive. Eppinger (1908a, b) reported the case history
of a girl aged 19 who fractured her skull and died as the result of jumping
out of a window a day following her admission to hospital for treatment of
a typical attack of catarrhal jaundice. A few days before the onset of

jaundice she had gastro-intestinal symptoms. At autopsy the liver was found to be normal macroscopically and on histological examination, but the mucosa of the stomach and duodenum was swollen and the papilla of Vater was prominent. The ostium of the common bile duct was blocked as a result of inflammation of its wall and hypertrophy of lymphoid tissue. Eppinger in fact taught that all jaundice was obstructive in origin, whether the obstruction occurred in the larger extraheptic ducts or in the biliary capillaries.

CAMPAIGN JAUNDICE

The view that catarrhal jaundice was due to mechanical obstruction at the entrance to the bile duct, or swelling of the mucous membrane of the bile duct, of the papilla of Vater and surrounding part of the duodenum was widely held until the late 1920s. This is surprising since it was known that epidemic jaundice occurred during the Middle Ages particularly during wars ("campaign jaundice"), and jaundice was followed closely in importance by plague and cholera as the cause of pandemics in Europe. The historical account of epidemic jaundice by von Bormann et al. (1943) mentions an outbreak in Germany in 1629 and reference is also made to an outbreak of jaundice which occurred in the British Army in Flanders in 1743. In 1764, Monro carefully documented jaundice in an account of diseases which were common in British troops in Germany from January, 1761 to March, 1763. Jaundice afflicted Napoleon's army in Egypt, although it is uncertain whether this was due to epidemic hepatitis because of the high mortality rate. During the American Civil War (1861–1865) numerous cases of jaundice were reported among the Federal troops, as shown in Table 1.1.

TABLE 1.1

Incidence of jaundice among the Federal troops during the American Civil War

Region	Number of men	Cases of jaundice	Deaths from jaundice
Atlantic	1 087 041	21 963	37
Central	1 101 758	20 497	124
Pacific	29 160	109	0

Epidemic jaundice occurred during the Franco-Prussian war in 1870 among the troops and among the civilian population during the siege of

Paris. The French referred to infective hepatitis as *"jaunisse des camps"* and the Germans as *"Soldatengelbsucht"*. During the Boer War in South Africa, 5648 cases of jaundice were recorded and the mortality was low. Large epidemics of jaundice occurred in the Japanese Navy during the war with Russia (1904–1905) and huge epidemics of hepatitis were recorded during the First World War particularly in the Middle East theatres. The following account is recorded by MacPherson *et al.* in the *History of the Great War*, Medical Services, Diseases of the War:

> Under the titles epidemic catarrhal jaundice of campaigns, epidemic jaundice of campaigns, and camp jaundice, is included a form of jaundice usually slight in degree in which the constitutional symptoms are mild. It has the features of an infection, either a blood infection which has localized in the duodenum, for example, or less often perhaps an infective gastritis which has extended to the duodenum. The usual symptoms are malaise, transient fever, headache, anorexia, nausea, abdominal discomfort, with jaundice supervening later. In France and Flanders these cases of jaundice only occurred singly or in small groups, and not in epidemics as they did in the Eastern theatres of war.
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> Epidemic catarrhal jaundice broke out in certain camps in Alexandria in July, 1915, and thereafter spread rapidly to Gallipoli, Mudros, Salonika, and ultimately to Mesopotamia, the Dardanelles. . . . The usual history in any battalion affected commenced with one or two isolated cases; then there was an interval of about three or four weeks with an occasional case; then a large number of cases for three weeks; and finally an occasional case for a few more weeks.

Martin (1918) noted that "during the autumn of 1915 troops at Gallipoli, and to a lesser extent in Egypt, suffered from a nearly non-fatal form of infectious jaundice, usually without complete obstruction of the bile passages, and not associated with any tendency to haemorrhages. It appeared to be markedly infectious; some units had 25% of their strength affected and many cases occurred amongst patients in hospital for other complaints". The epidemics of jaundice during the Second World War attained vast proportion: 200 000 cases occurred in U.S. troops from 1942 to 1945 (Havens, 1961) and over 5 000 000 cases occurred in the German armies and civilians alone according to Gutzeit (1950), whilst huge epidemics swept through the Allied Forces especially in the Mediterranean region (Cullinan, 1952). Indeed the number of cases was so large as to influence the strategy of war. Despite the remarkable advances in knowledge of the epidemiology of hepatitis this infection was again a serious

problem in the Israel War of Independence in 1948, the Arab–Israel conflicts in 1956 and 1967, during the Korean campaigns and in more recent years in South Vietnam.

THE VIRAL AETIOLOGY OF HEPATITIS

The view that catarrhal jaundice was not due to mechanical obstruction of the extrahepatic biliary tract, despite the presence of inflammation of the upper gastro-intestinal tract, was first suggested by Stokes (1834). This was subsequently supported by the work of Heitler in 1887 and Flindt (1890), who considered that this was a generalized infection which reached the liver parenchyma through the blood stream. It was also concluded that hepatitis might occur both in sporadic and epidemic form and that it was intimately associated with acute and subacute necrosis of the liver. Bacteriological investigations of epidemic jaundice were carried out with great persistence by many investigators. Occasionally *Salmonella typhi* and *S. paratyphi* were isolated as well as a variety of other bacteria including *Bacillus coli communis*. Spirochaetal infection as a cause of epidemic hepatitis was definitely excluded once leptospirae were identified.

McDonald (1908, 1918) summarized the views held at the time from a study of acute yellow atrophy or acute necrosis of the liver, and from a series of subacute cases and stated: ". . . that there is much to be said in favour of the view that in the causation of acute yellow atrophy we have two factors at least at work. It may well be that the typical condition is only produced when some special virus acts on a previously damaged liver". Cockayne (1912) considered that in catarrhal jaundice the liver is probably infected by the blood stream and the infection may spread down the duct. He also pointed out the great rarity of gastro-intestinal trouble without jaundice in epidemics and that the early appearance of tenderness over the liver suggests that the liver is attacked primarily. Cockayne concluded that sporadic and epidemic catarrhal jaundice and acute yellow atrophy of the liver are due in the great majority of cases to the same cause, a specific organism of unknown nature. The word "virus" appears once in the discussion. Similar observations were reported by Martin in 1918, and he also commented that in discussing cases of jaundice in Alexandria in November 1915, "Professor Kartulis, using various microscopical and bacteriological methods, searched for the pathogenic agent in both classes of infectious jaundice, but hitherto in vain. He suggests that *it must be invisible like that of yellow fever.*" The viral aetiology of infective

hepatitis gained support from then onwards (Stokes *et al.*, 1920; Bergstrand, 1930; Findlay *et al.*, 1931; Findlay and Dunlop, 1932). The aetiology of infective hepatitis was reviewed by Findlay *et al.* (1939) who pointed out that in the absence of a laboratory animal which can be infected with hepatitis, the viral aetiology of the disease would have remained hypothetical had it not been for the occurrence of hepatitis following immunization against yellow fever. The human volunteer studies during the Second World War and subsequently, finally established the viral aetiology of epidemic hepatitis.

THE EMERGENCE OF A SECOND TYPE OF HEPATITIS

The history of hepatitis type B (serum hepatitis) is much shorter. Lurman (1885) reported the earliest recognized epidemic of serum hepatitis among the shipyard workers in Bremen in 1883. Some cases of smallpox occurred in Bremen and extensive vaccinations were carried out with glycerinated lymph of human origin. Of the 1289 vaccinated employees, 191 developed jaundice after intervals of several weeks to 8 months. Several hundred workers employed after the vaccination had been completed and those inoculated with different batches of "lymph" were not affected. Lurman's paper is a classical example of careful epidemiological observations. MacCallum (1972b) pointed out that it is strange that no record of other events such as those described by Lurman had been found earlier in view of the practice of using human lymph for smallpox vaccination and the widespread practice of tattooing. The wide-scale introduction and common use of large syringes and long needles with the advent in 1909 of salvarsan therapy in venereal disease clinics was soon followed by sudden outbreaks of jaundice. The drug was naturally suspected as a potential hepatotoxin. The Salvarsan Committee of the Medical Research Council, which published two reports in 1919 and 1922, could come to no decision on the precise cause of jaundice, but toxicity of special batches of arsenobenzol was excluded. It is of interest that in 1917 there was an outbreak of jaundice with 15 deaths at the venereal disease department of Cherryhinton Military Hospital, Cambridge and the MRC committee drew attention to the fact that at the same time there was a small epidemic of jaundice among the children in an elementary school near by, affecting 15 children and one adult. Outbreaks of jaundice were subsequently reported on numerous occasions in venereal disease clinics. Murray (1930) reported

that jaundice occurred in 11% of 118 soldiers, 60–126 days after intra-venous injection with acriflavine for the treatment of gonorrhoea. Of interest is that human serum was added to acriflavine since it was believed that the bactericidal activity of acriflavine was enhanced by the added serum. Ruge (1932) reported that among 2459 patients with jaundice in the German Navy between 1919–1929, 700 cases followed treatment with arsenic for syphilis. The jaundice was attributed to the hepatotoxicity of arsenic. Soffer (1937) recorded jaundice among patients treated with bismuth injection for syphilis and ascribed this to the toxicity of bismuth and neoarsphenamine on the liver. MacCallum *et al.* (1952) in recording the problem of infective hepatitis in the *History of the Second World War* wrote as follows:

> ... In the years 1939–45, hepatitis, a known hazard of arsenotherapy, became a much more serious problem in certain clinics when large numbers of patients were having venepuncture done and receiving subcutaneous, intramuscular or intravenous injections of therapeutic substances. This was the case in certain diabetes-clinics, sanatoria, and arthritis-clinics, but it was particularly true in the venereal disease treatment clinics for Service personnel. Here the hepatitis was confined to syphilitic patients who for some months had been receiving weekly intravenous injection of arsenical preparations. (Before the injection was made, a few drops of blood were aspirated into the syringe to ensure that the needle was in the vein.) In each of these venereal disease treatment centres, a practicable system had had to be improvised quickly to deal with a large stream of men attending for intravenous therapy. Apparatus was scarce and syringes were easily broken during boiling, so the sterilization technique between patients was usually reduced to a thorough wash of the blood-contaminated syringe in running water. This was generally assumed to be adequate in the particularly difficult circumstances, as no septic complications were noted and the possibility of syringe transmission of diseases other than sepsis was scarcely considered. The incidence of hepatitis gradually rose to 30 per cent of syphilitic patients and in certain centres was about twice the figure. From observations of the existing conditions, it was suggested by Bigger and by MacCallum that this hepatitis was being transmitted in the course of venepuncture and intravenous injections. It soon became clear that this explanation was the correct one.
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>   On investigation, it was found that the incidence of hepatitis tended to be very low in venereal disease clinics where syringes were sterilised between patients, whereas it tended to be high in clinics where the syringes were merely washed. ...

But outbreaks of jaundice were not restricted to venereal disease clinics. Flaum *et al.* reported in 1926 epidemic jaundice in a diabetic clinic and Sherwood (1950) recorded syringe-transmitted hepatitis in four of nine patients with diabetes treated in a hospital. A common syringe was used for the administration of insulin. Droller (1945) previously observed hepatitis among diabetics, but on that occasion it was traced to a common syringe which was used for the withdrawal of blood for blood sugar estimations. Hartfall *et al.* reported in 1937 hepatitis, indistinguishable from catarrhal jaundice, in 85 of 900 patients treated for rheumatoid arthritis, with injection of gold. However, it was not until 1943 that a common factor was suggested by MacCallum in all these outbreaks, namely that jaundice may be transmitted from patient to patient by means of syringes which had been inadequately sterilized between injections.

MacNalty (1938) reported that between 82 and 109 persons were inoculated with a batch of convalescent measles serum that caused jaundice in 41 people out of whom eight died. These cases were scattered over a wide area in the south of England. A batch of measles adult serum pooled and Berkefeld candled in the same laboratory, gave rise to at least 11 cases of jaundice with one death. Other batches of measles serum were also suspect. In the same year, Propert observed that a number of children in an institution developed hepatitis 60 days after the injection of human convalescent measles serum. The term "homologous serum jaundice" came into use in Britain after the publication of a Ministry of Health memorandum in 1943 describing the outbreak of jaundice and deaths which followed the subcutaneous administration of measles convalescent serum to children. Earlier, Findlay and MacCallum (1937) drew attention to the occurrence of jaundice following yellow fever immunization and they considered that the jaundice may have been due to some organism injected with the vaccine or serum. However, it was considered that if a hypothetical virus pathogenic for man were directly injected with the inoculum it was surprising that under 3% of persons developed symptoms. Although the presence of a virus could not be entirely excluded the evidence against it appeared to be substantial. Nevertheless, it was concluded that the occurrence of jaundice 2–6 months after yellow fever immunization was analogous to the occurrence of outbreaks of jaundice following anti-syphilitic treatment or injections of acriflavine. In October, 1939, about 27% of a group of 304 persons inoculated with one lot of yellow fever vaccine developed jaundice 4 months after injection. In May,

1940, more cases of jaundice appeared in South America in relation to a small number of different lots of vaccine. Out of 107 000 people, 1072 developed jaundice 12–20 weeks following injection with the incriminated batches of vaccine. However, the largest outbreak of serum hepatitis occurred in 1942 when 28 585 young American soldiers inoculated with yellow fever vaccine developed jaundice and 62 of them died (Annotation, 1942). There was considerable suggestive evidence that hepatitis and jaundice following yellow fever immunization was due to a filterable agent present in the human serum incorporated in the serum-Tyrode virus culture medium containing minced chick embryo (Findlay and MacCallum, 1937). In 1939, Findlay *et al.* concluded ". . . that pools of apparently normal human serum should not be used for inoculation unless the medical history of all the donors can be followed over a considerable period of time, preferably at least one month, the probable incubation period of infective hepatitis . . .".

Findlay and MacCallum (1937) also pointed out the similarities between jaundice following yellow fever immunization and acute liver necrosis of horses, which was known as "staggers" in South Africa, reported by Theiler (1919). This condition occurred 62–78 days after inoculation of homologous serum against horse-sickness. In England, 15 ml of lamb dysentery horse serum prepared early in 1935 was injected subcutaneously into 617 horses. About 6 weeks later some of the horses developed a staggering gait and eventually complete paralysis. Most of the affected animals showed well-marked jaundice of the visible mucous membranes. In 182 (30%) of the inoculated animals the disease was mild; 7·6% of the 716 inoculated horses died and yellow atrophy of the liver was a prominent feature at autopsy. In Montana, 5193 horses were treated with either 20% suspension of guinea pig brain infected with equine encephalomyelitis with horse serum as a diluent, or with serum alone. After a period of 32–89 days post-injection, 89 animals became ill and 79 died. Jaundice of the mucous membranes was noted. In Norway, anti-anthrax serum was produced in horses and cows, and either homologous or heterologous serum was injected. Acute or subacute necrosis of the liver occurred in the horses receiving homologous serum mostly after an interval of 50–60 days. Altogether 101 horses were affected and 50 died. Inoculated cows were not affected (Memorandum, 1943). It is interesting to note that besides hepatitis, there was diffuse enteritis and renal damage.

A CHURCHILLIAN STORY

An incident with which F. O. MacCallum was concerned is best told in his own words:

> One day in 1942, I received a message to go to Whitehall to see one of the senior medical advisers and when I arrived I was asked "What is this yellow fever vaccine and how dangerous is it?" After explaining its constitution and the possibility of a mild reaction four to five days after inoculation I was told that the Cabinet was at that moment debating whether or not Mr Churchill should be allowed to go to Moscow, which he wished to do in a few days' time. The yellow fever vaccine inoculation was theoretically essential before he could fly through the Middle East, but I explained that no antibody would be produced before 7 to 10 days so that there would be little point in giving the vaccine. It was finally decided that the vaccine would not be used, and the administrators would take care of the situation. Several months later, I received an irate call from the Director of Medical Services of the R.A.F., who had been inoculated from the same batch of vaccine which would have been used for Mr Churchill, and was informed that the D.G. had spent a very mouldy Christmas with hepatitis about 66 days after his inoculation. This was the first I knew that we were in for trouble again with our vaccine after a lapse of five years. Unfortunately, owing to the war, I had never received the information that it had been found in Brazil that serum was not necessary for stabilisation of the 17D virus in the vaccine. I will leave you to speculate on what might possibly have been the effect on the liver of our famous statesman and our ultimate fate if he had received the icterogenic vaccine (MacCallum, 1972b).

Jaundice also occurred after the inoculation of vaccines other than yellow fever vaccine. Sergiev et al. (1940) reported jaundice following the use of sand-fly fever vaccine in Russia. Morgan and Williamson (1943) described nine cases of jaundice which developed 7 to 16 weeks after transfusion with serum or plasma and which resembled closely the hepatitis following inoculation against yellow fever and measles. Other reports soon followed including hepatitis after the administration of mumps convalescent plasma (Beeson et al., 1944). Beeson (1943) was the first to describe jaundice after the transfusion of whole blood and he pointed out that the real frequency of hepatitis as a complication of transfusion will only be known when there is a concerted effort to recognize such cases. Transmission of the virus of hepatitis by blood transfusion thus became

recognized as well as the risk of hepatitis associated with the use of pooled and dried human plasma and human blood products (reviewed by Zuckerman, 1970, 1975a).

HUMAN EXPERIMENTATION

The viral aetiology of hepatitis was finally established during the Second World War by successful transmission experiments to human volunteers first in Germany by Voegt (1942), in the British Mandate of Palestine by Cameron (1943) and later by more extensive studies carried out in Great Britain (reviewed by MacCallum *et al.*, 1951) and in the United States (reviewed by Havens, 1947, 1963). The following account by MacCallum *et al.* (1952) in the *History of the Second World War* is of interest:

Shortly after the arrival of United Kingdom troops in Palestine and Egypt in 1940 infective hepatitis again appeared. As previously, the cause of the disease was completely unknown, and the earliest investigation of Cameron (1943) showed that whereas it was common among troops entering the country, cases were apparently rare among the local adult population of Palestine. Intensive investigations by Cameron and others at the 23rd (Scottish) General Hospital at Sarafand failed to shed any light on the mode of transmission of infection, neither was it possible to isolate any cultivable bacterium with regularity from patients or from post-mortem material; animal inoculation tests also proved to be negative. Faced by such difficulties Cameron injected six human volunteers intravenously with infected blood, successfully reproducing the condition in these men, and thereby demonstrated the artificial communicability of the disease. Following the departure of Cameron to India Command, laboratory investigations were renewed in 1941 by van Rooyen and Gordon (1942) at the 15th (Scottish) General Hospital, Agouza, Cairo, with special reference to isolation of an infective agent of the virus type. A wide range of laboratory animals including monkeys, baboons, pigs, gerbilles, jerboas, and desert rodents indigenous to these parts of the world were inoculated by numerous different routes—with the co-operation of the late Dr John Bland of the Giza Ophthalmic Research Laboratories—but without success. In view of the many thousands of cases which had occurred, and loss of manpower, in the text of an official report on the subject van Rooyen in 1942 proposed to the D.D.P., Colonel J. S. K. Boyd, that higher authority be sought for the performance of human inoculation tests at Abassia Military Detention Barracks. The proposal received the attention and support of Professor L. J. Witts, Chairman of the Infective Hepatitis Committee of the Medical Research Council, who visited the site of the proposed experiment

in 1944; but it was impossible to carry out human transmission in Egypt on Service patients. Simultaneously, the United States Army Commission on Respiratory Diseases were studying the occurrence of the disease in American forces in Egypt and Sicily and Italy. Special attention was paid to an agent of the virus group, having in mind the possibility of spread by intestinal content, occurring in fatal cases among British and American troops in Egypt (Paul, Havens, and van Rooyen, 1944). The need for human volunteers was immediately appreciated, stools were collected from American troops and arrangements made for collection of similar material from British soldiers. In Britain, MacCallum and Bradley (1944) performed similar experiments and successfully reproduced infective hepatitis, after an incubation period of twenty-seven to thirty-one days, in human cases of rheumatoid arthritis by feeding them with faecal material. The results of Havens *et al.* (1945) also showed that the infective agent was transmissible by faeces and the incubation period could be as short as twenty to twenty-two days. . . .

Inevitably there are many limitations to this type of investigation, but nevertheless the studies in volunteers have furnished considerable valuable information about the mode of transmission, the infectivity as well as some of the physical and chemical properties of the viruses causing hepatitis. Many of the early studies were carried out mainly with volunteers who were conscientious objectors during the war, prisoners, volunteer patients with rheumatoid arthritis and mentally handicapped children at the Willowbrook State School in New York. It should be noted that the studies at Willowbrook have been conducted in accordance with the World Medical Association's Draft Code of Ethics on Human Experimentation (Krugman *et al.*, 1967, 1971).

HEPATITIS A AND B

During the early period of transmission experiments it was difficult to distinguish clearly between infectious hepatitis and serum hepatitis in the absence of specific laboratory tests. Soon, however, differences between the two types of hepatitis became apparent, based essentially on epidemiological observations, particularly the route of infection and the incubation period. The terms hepatitis A, for infectious or epidemic hepatitis, and hepatitis B, for serum hepatitis or homologous serum jaundice, were introduced by MacCallum in 1947 and generally adopted in 1973 by the Scientific Group on Viral Hepatitis of the World Health Organization and by the Expert Committee on Viral Hepatitis in 1977.

Additional information and confirmation of earlier results were obtained from the studies carried out since 1956 at the Willowbrook State School, New York, an institution for mentally retarded children. The patient population at this institution increased from 200 children in 1949 to over 6000 in 1963 and viral hepatitis has been an endemic disease amongst the children since 1953. Since 1956, 1153 cases of viral hepatitis with jaundice were observed to have been transmitted by natural contact in this institution. Most of the newly admitted children acquired the infection within the first 6 to 12 months of admission to the institution. During a period of some 12 years approximately 250 children had participated in experimentally induced hepatitis at the Willowbrook State School.

The studies at Willowbrook confirmed that there are two distinct epidemiological, clinical and serological types of viral hepatitis. One type of illness, induced by MS-1 serum, resembled closely classical hepatitis A. It was characterized by a short period of incubation of about 4 weeks and it was highly infectious by natural contact. The results of biochemical tests were also characteristic of hepatitis A. The second type of infection, induced by MS-2 serum, resembled hepatitis type B. This infection had a long incubation period and biochemical tests of liver function were, on the whole, more typical of the findings in serum hepatitis. It was also observed, contrary to the generally accepted view, that the serum hepatitis-like infection could be transmitted by the oral route, although it was less infectious even for close contacts. Other experiments showed that the children inoculated with MS-1 serum had homologous immunity but there was no cross-immunity between MS-1 infection and the type of infection caused by MS-2. This apparent lack of heterologous immunity between the two types of hepatitis confirms the volunteer experiments conducted during the 1940s and early 1950s in Great Britain and the United States.

The studies in experimentally infected volunteers also revealed the presence of viraemia during the long incubation period of hepatitis B, namely 87 days before the onset of hepatitis (Neefe et al., 1944b), 60 days and 16 days before the appearance of jaundice (Paul et al., 1945; Havens, 1963). Evidence that a prolonged carrier state for as long as 5 years may result in some patients with or without signs of liver disease was provided by Stokes et al. (1954), Neefe et al. (1954) and Murray et al. (1954). Zuckerman and Taylor demonstrated in 1969 persistent carriage of hepatitis B antigen for over 20 years in the serum of a well-documented former volunteer blood donor. These observations thus confirmed long-

held epidemiological and clinical impressions that a prolonged carrier state of hepatitis B virus may occur in the blood of at least some people. With the development of sensitive laboratory techniques for the detection of antigens and antibodies associated with hepatitis B virus it is conservatively estimated that there are about 176 million carriers of hepatitis B surface antigen in the world today.

SPECIFIC SEROLOGICAL TESTS FOR VIRAL HEPATITIS

Havens (1954) reviewed the attempts to develop specific serological tests for viral hepatitis and referred to the work of J. S. H. Gear in the investigation of the cases of hepatitis following yellow fever vaccination of American troops in 1942. During the course of those investigations Gear showed that a substance, presumably an antigen, present in the acute phase serum of patients with serum hepatitis and later found also in serum collected during the incubation period reacted in a precipitin test with a substance, presumably an antibody, present in the convalescent serum. In these tests the neat convalescent serum, presumably containing the antibody, was added to precipitin tubes containing serial dilutions of the acute phase serum. A drop of the neat convalescent serum placed inside the mouth of the precipitin tube flowed to the bottom to form an interface with the diluted acute phase serum above, and the precipitin reaction took place at the interface and with proper illumination could be clearly observed. The substance detected in the acute phase serum appears to be similar to the antigen now called Australia antigen (Gear, 1948, 1972 and personal communication). Similarly, precipitating and complement-fixing antibodies were demonstrated in convalescent phase sera which reacted with antigen(s) in acute phase sera and in saline extracts of normal human liver and liver from patients with hepatitis (Sawyer *et al.*, 1944; Eaton *et al.*, 1944). Pollard and Bussell (1953) also described a substance in the acute phase serum of a patient with hepatitis B which fixed complement with sera from patients convalescent from hepatitis B, but not with sera from patients recovering from hepatitis A or with other forms of jaundice. However, it was not until the discovery of Australia antigen (hepatitis B surface antigen) that a specific and reproducible serological test finally became available for the diagnosis of hepatitis type B.

DISCOVERY OF AUSTRALIA ANTIGEN

Polymorphism is defined as the occurrence in the same habitat of two or more inherited forms of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation. In polymorphic traits two or more of the genotypes determining variation of the trait are common in the population. Polymorphisms are believed to arise as a result of selective differences between genotypes and they provide convenient systems for the study of inherited discontinuous biochemical variation in humans. Included in such systems are the red blood cell antigens (ABO, MNS, P, Rhesus, etc.), sickle cell haemoglobin, haptoglobin, transferrin, glucose-6-phosphate dehydrogenase deficiency, gammaglobulin groups and so on. Allison and Blumberg (1961) argued on this basis that patients who are transfused would be likely to receive blood containing proteins which they had not inherited or acquired since donor blood is commonly only typed for the major red blood cell antigens. Some of these differences might, therefore, be antigenic and lead to the development of antibodies in the transfused patients. A systematic investigation of the serum of transfused patients was begun using the two dimensional micro-Ouchterlony immunodiffusion technique to visualize the lines of precipitation.

After the examination of some 13 sera from transfused patients in the centre well and a panel of sera from different geographical areas in the peripheral wells, one serum from the transfused patients was found to contain a precipitin which reacted with some of the sera in the panel. It was soon demonstrated that this antiserum defined a system of inherited antigenic specificities of the low density β-lipoproteins, designated the Ag system. A search for additional such systems was initiated and in 1963 the two sera from multiply transfused American haemophiliacs were found to give a single precipitin line with only one of 24 sera in the test panel. The antigen in this single serum contained little or no lipid and clearly differed from the Ag precipitin, but it stained with azocarmine indicating that protein was a major component. Since the reacting serum was obtained from an Australian aborigine, the antigen was named "Australia" antigen (Blumberg et al., 1965). Subsequent studies on the distribution of the antigen in normal populations in different geographical areas of the world revealed that this antigen was very rare or absent in normal North American and European communities but that it occurred frequently in the serum of apparently healthy people living in the tropics

and Southeast Asia (6–25%). The antigen was found frequently in the serum of patients with acute leukaemia and it was suggested in 1965 that the presence of Australia antigen may be of value in the early diagnosis of leukaemia and further that the antigen may be related to the virus postulated as the cause of leukaemia (Blumberg, 1968). Evidence also suggested that Australia antigen was inherited as a simple autosomal recessive trait. A corollary of this hypothesis was that individuals at high risk of developing leukaemia have an increased frequency of persistent Australia antigen. Patients with Down's syndrome are known to have a high risk of leukaemia and up to 30% of the sera from such patients were found to contain Australia antigen (Blumberg et al., 1967; Sutnick et al., 1968).

In 1966 one of Blumberg's patients with Down's syndrome, who on initial examination did not carry the Australia antigen but in whose serum the antigen was found on a second test, was investigated further. Because many serum proteins are manufactured in the liver, biochemical liver function tests were carried out. These investigations and a liver biopsy revealed that this patient developed anicteric hepatitis concomitant with the appearance of Australia antigen in his blood. In April, 1967, a technician working with Australia antigen in Blumberg's laboratory developed malaise, loss of appetite and dark urine. Australia antigen was detected in her blood for only a single day and she subsequently developed a mild icteric hepatitis (Blumberg, 1968). Sera of patients with viral hepatitis were also examined for Australia antigen and the antigen was found in 10·4%.

Another group of investigators was led by Prince, and in 1964, Prince et al. described the finding of an antigen in foci of hepatic parenchymal cells in nine out of 32 liver biopsies obtained from patients with anicteric hepatitis. The antigen was detected by a complement-staining fluorescent antibody technique in the nuclei and cytoplasm of the cells or in either of these sites. Antibody to this antigen was found in a pool of serum from cases of acute hepatitis with jaundice among soldiers in Korea, in sera of all patients with the antigen and in the serum of one American patient with probable hepatitis B acquired in Denmark. This antibody was not detected in a variety of control sera, and the antibody concerned did not stain a large number of control human liver tissues with non-viral hepatic necrosis. The findings were interpreted at the time as representing hepatitis B virus, but the significance of these observations was not appreciated until the application of the immunofluorescent antibody technique for the localization of Australia antigen (hepatitis B surface

antigen) and hepatitis B core antigen. Prince (1968), using methods closely similar to those employed by Blumberg and his group, detected an antigen which he termed the SH (serum hepatitis) antigen, in the serum of patients during the incubation period and early acute phase of post-transfusion hepatitis, in patients with serum hepatitis and in the serum of eight out of 4844 volunteer blood donors. The SH antigen was not found in the serum of patients with infectious hepatitis. At about the same time, a strong association between Australia antigen and post-transfusion hepatitis was observed by Okochi and Murakami (1968) in Japan. It soon became evident that Australia antigen and the SH antigen were identical and subsequent studies established the relationship between the antigen and hepatitis B. A new era in hepatitis research began, resulting from an accidental discovery of a circulating antigen in the serum of an Australian aborigine, and in 1976 the Nobel Prize in Physiology or Medicine was awarded to Baruch S. Blumberg.

2

Nomenclature of hepatitis viruses and their antigens

The terminology of viral hepatitis has numerous synonyms, and the discovery of Australia antigen and subsequently other antigens was followed by the introduction of a number of terms describing identical antigen–antibody systems and the use of complex and on occasion almost bizarre abbreviations. It became desirable to arrive at an easier, more acceptable and uniform terminology to lessen the confusion which was developing in a rapidly advancing field and the nomenclature was revised by the World Health Organization in the light of recent findings in 1973 and 1975.

It was proposed that the common forms of viral hepatitis be referred to as hepatitis A and B, terms introduced by F. O. MacCallum in 1947. Hepatitis A replaces therefore such synonyms as infectious hepatitis, epidemic jaundice, catarrhal jaundice; and hepatitis B is the recommended term for serum hepatitis, homologous serum jaundice, syringe jaundice, long-incubation hepatitis and so on.

The WHO Expert Committee on Viral Hepatitis (1977) suggested the following modifications in nomenclature which took into account recent findings from many laboratories:

Hepatitis A virus
HAV   Hepatitis A virus. A small virus in the range of 25 to 28 nm
           possessing cubic symmetry. Full and empty particles exist.
           Both full and empty particles are identified by immune
           electron microscopy. Serological tests for hepatitis A virus

include complement fixation, immune adherence haem-
agglutination, radioimmunoassay and enzyme immunoassay.

Anti-HAV   Antibody to hepatitis A virus.

Hepatitis B virus

HBV   Hepatitis B virus. A 42-nm double-shelled virus, originally
            known as the Dane particle.

HBsAg   Hepatitis B surface antigen, originally known as Australia
            antigen. The hepatitis B antigen found on the surface of the
            virus and on the accompanying unattached 22-nm spherical
            particles and the tubular forms.

HBcAg   Hepatitis B core antigen. The hepatitis B antigen found within
            the core of the virus.

HBeAg   The *e* antigen which is closely associated with hepatitis B
            infection.

Anti-HBs   Antibody to hepatitis B surface antigen.

Anti-HBc   Antibody to hepatitis B core antigen.

Anti-HBe   Antibody to the hepatitis B *e* antigen.

Progress in the specific diagnosis of viral hepatitis has proceeded to such
an extent that it has revealed a new type of hepatitis which is unrelated
to hepatitis A or B virus (non-A: non-B hepatitis). This new form of
hepatitis is now the most common type of post-transfusion hepatitis
occurring in some areas. In addition, sporadic cases of hepatitis occur
which are also unrelated to hepatitis A or B viruses. However, precise
virological criteria for this new form of hepatitis are not yet available.

SUBDETERMINANTS OF HEPATITIS B SURFACE ANTIGEN

Careful serological analysis revealed that the particles bearing hepatitis B
surface antigen activity share a common group specific antigen *a* and the
particles generally carry at least two mutually exclusive subdeterminants
d or *y* or *w* and *r*. The subtypes are the phenotypic expressions of distinct
genotype variants of hepatitis B virus. Four principal phenotypes have
been recognized, *adw*, *adr*, *ayw* and *ayr* but other complex permutations of
these subdeterminants and new variants have been described, all apparently
on the surface of the same physical particles. A remarkable geographical
pattern of distribution of hepatitis B subtypes has emerged with four
global zones, where there is an excess of one subtype and regions where a
mixture of subtypes is common. These subtypes provide valuable epi-

demiological markers and offer a method for distinguishing one of several sources of infection. The different subtypes are not associated with particular clinical forms of liver disease. Eight distinct categories of hepatitis B surface antigen and two categories of mixed subtypes have been recognized, consisting of various combinations of the subdeterminants d, y, w and r, and other variants originally described as being related to the group determinant a. The latter, however, are better designated as variants of w since they always behave as alleles of r. The terminology of the subdeterminants is, therefore, as follows:

$aywı$	(a_1yw)	$adw2$	(a_2^1dw)
$ayw2$	(a_2^1yw)	$adw4$	(a_3dw)
$ayw3$	(a_2^3yw)	adr	
$ayw4$	(a_3yw)	$adyw$	
ayr		$adyr$	

The major subdeterminants behave as though they comprise two allelic groups: d and y on the one hand, and $wı$, $w2$, $w3$, $w4$ and r on the other. But these systems are probably not completely independent, since only two of the four variants of w (found with y) have been demonstrated with d. The two mixed subtype categories are rare and may possibly result from phenotypic or genotypic mixing of determinants during simultaneous infection with viruses associated with more than one subtype of the surface antigen (WHO, 1977).

Other surface antigenic reactivities, such as q, x, f, t, j, n and g, have also been described. The necessary serological comparisons between these antigenic reactivities have not yet been made (reviewed by Le Bouvier and Williams, 1975).

SUBDETERMINANTS OF HEPATITIS B e ANTIGEN

Another marker of hepatitis B virus infection is the e antigen. This antigen is a soluble antigen which differs immunologically from the other subdeterminants of the surface antigen and it also has different physico-chemical properties. The antigen seems to be specific for hepatitis B, since it is only found in sera containing hepatitis B surface antigen. The e antigen appears to correlate with the number of circulating virus particles, the degree of infectivity of surface antigen positive sera and the severity and course of chronic liver disease. Two antigens have been identified and are designated HBeAg/1 and HBeAg/2. A third component of e antigen, e_3, has been identified more recently (see Chapter 10).

The epidemiology of viral hepatitis

HEPATITIS A

Incidence

Morbidity rates of hepatitis A are based on notification and statistical records; Sweden and Denmark have kept statistical records of hepatitis with jaundice since 1928. Viral hepatitis became notifiable in the United States of America in January 1952, and since 1966 hepatitis A and hepatitis B have been notifiable separately. Hepatitis has also been reported for a number of years in many European countries and in June 1968, the notification of infective jaundice became compulsory in England and Wales. Thus statistical records of morbidity and mortality are available for a number of countries. However, no account is taken of subclinical infections and anicteric cases, which occur with a generally accepted rate of

some 10 cases or more for each patient with clinical jaundice. Notifications tend to be extremely variable, not only from city to city and from region to region but also with a number of poorly definable factors such as holiday seasons, physician awareness and so on. Furthermore, the degree of under-reporting is believed to be very high and it is doubtful whether more than perhaps 50% of cases of jaundice are actually notified.

Age incidence

All age groups are susceptible to hepatitis. Until recent years the highest incidence in the civilian population was observed in children of school age but in a number of countries, including Sweden and the U.S.A., as many as 60–70% of the notified cases occur in adults. This shift in age incidence is reminiscent of the change of age incidence which occurred with poliomyelitis during and after the Second World War, reflecting improvement in socio-economic conditions.

Seasonal pattern

In temperate zones the characteristic seasonal trend is for a marked rise in incidence in the autumn and early winter months with a rapid and progressive fall from January to a minimum during midsummer. Mosley (1972) noted, however, that since 1966 the seasonal peak has been lost in the U.S.A.

Mode of spread

Hepatitis A is spread by the intestinal–oral route, most commonly by close contact, and infection occurs readily in conditions of poor sanitation and overcrowding. Food or waterborne transmission is not a major factor in the maintenance of this infection in the developed countries. Ingestion of shellfish cultivated in polluted water is associated with a high risk of acquiring hepatitis A. This infection is also not infrequently acquired by travellers to areas of high endemicity. Outbreaks of hepatitis A have also been described in handlers of newly captured non-human primates. Hepatitis A is not transmitted by blood and blood products and rarely if ever by the parenteral route (Szmuness *et al.*, 1977; Papaevangelou *et al.*, 1978), although this has been achieved experimentally in volunteers.

Incubation period

The incubation period of hepatitis A is between 3 and 5 weeks and most commonly 4 weeks. This period of incubation has been confirmed by

human volunteer experiments and by transmission to susceptible chimpanzees. The incubation period of the MS-1 strain of hepatitis A transmitted either by the oral route or parenterally to volunteers was between 35 and 50 days.

HEPATITIS B

Hepatitis B has been defined in the past on the basis of infection occurring in patients about 40–200 days after the injection of human blood or plasma fractions. However, the mode of spread of hepatitis B continues to present intriguing and unexpected findings. The huge reservoir of hepatitis B virus in the human population with a prevalence of hepatitis B surface antigen among apparently healthy adults varying from about 0·02–0·1% in parts of Europe, North America and Australia, over 5% in parts of eastern and southern Europe, the Middle and Far East, to as many as 20% in some tropical areas, poses a major public health problem. As sensitive laboratory techniques for markers of hepatitis B infection were developed the epidemiological concepts of this infection have undergone significant changes, and it is now clear that the direct inoculation of blood and certain blood products cannot be the principal method of spread of the infection, nor account for the some 176 million carriers of hepatitis B. The question of whether an apparently healthy carrier can transmit frequently the infectious agent by means other than blood is thus of great importance and much has been written on the subject. Hepatitis B surface antigen, a marker of the virus, has been found repeatedly in blood and in a variety of body fluids including saliva, menstrual and vaginal discharges, seminal fluid, amniotic fluid, colostrum and breast milk. The presence of the antigen in urine, bile, faeces, sweat and even tears has also been reported but not always confirmed. It is therefore not surprising that contact-associated hepatitis B is apparently of major significance.

Hepatitis B surface antigen in various body fluids other than blood
Hepatitis B surface antigen has been detected repeatedly in blood and certain plasma fractions, saliva, semen, breast milk, amniotic fluid and in certain exudates including pleural effusions and ascitic fluid. The antigen has also been reported in various tissue and body fluids contaminated with blood. Occasionally, the surface antigen was reported in bile and urine and a variety of other fluids including sweat and tears but confirmation of these is still awaited.
Saliva. Hepatitis B surface antigen has been detected in the saliva and

mouth washings obtained from patients with acute hepatitis B, from persistent carriers and from patients with chronic liver disease and anti-genaemia. Although occult blood was found in many samples of saliva, the presence of antigen in saliva was independent of the presence of blood. The titre of antigen in saliva is always low but tends to be higher in specimens containing occult blood. The antigen was never detected beyond 4 weeks after the onset of symptoms in acute hepatitis B and its finding in saliva was related to the titre of antigen in the blood (Lun Wong *et al.*, 1976). In persistent carriers the antigen was found intermittently in repeated saliva specimens (Feinman *et al.*, 1975).

Bancroft *et al.* (1977) collected a pool of whole-mouth saliva from five carriers of hepatitis B surface antigen. Occult blood was present in the saliva and immune electron microscopy revealed all the morphological entities associated with hepatitis B including the complete virus. Two gibbons (*Hylobates lar*) were injected subcutaneously with a small quantity of the saliva pool every other day for 3 days. One gibbon developed antigenaemia for 2 weeks accompanied by elevated alanine amino-transferase 12 weeks after inoculation. Surface antibody was detected in all subsequent serum samples. The second gibbon had elevated alanine and aspartate aminotransferases, 10 weeks and 4 days after exposure, and surface antibody was detected at 22 weeks. Eight gibbons were exposed to the saliva pool by the oral and nasal routes and by spray and brushing of teeth or by ingestion of saliva injected into a banana, but none were successfully infected. Thus although saliva was demonstrated to be infective, this was not achieved by a natural mode of transmission.

Alter *et al.* (1977) inoculated intravenously into seronegative chim-panzees saliva and semen containing hepatitis B surface antigen obtained from individuals clinically implicated in non-percutaneous transmission of this infection. Occult blood was not detected in the inocula. One chimpanzee inoculated sequentially into separate veins with saliva from three individuals developed acute hepatitis B. Surface antigen, *e* antigen, core antibody and surface antibody were detected in this chimpanzee and liver biopsies showed acute hepatitis that subsequently resolved. A second chimpanzee inoculated with semen developed the surface antigen and elevated serum alanine aminotransferase 4 weeks after inoculation and then died suddenly from an unidentified cause.

Although infectivity of saliva and semen was demonstrated it is noted that it was not shown that saliva and semen are infectious by their natural routes, namely by oral and/or venereal contact.

Semen. Examination by radioimmunoassay of specimens of saliva and seminal fluid from adult male carriers of hepatitis B surface antigen and males with acute or chronic liver disease associated with hepatitis B revealed the presence of antigen in 18 out of 24 saliva specimens and in 10 out of 19 samples of semen. The 10 men with antigen in the semen also had positive saliva samples. It is possible that some saliva samples were contaminated with traces of blood, but this is much less likely to have occurred with the semen specimens. The amounts of antigen in the saliva and seminal fluid were small compared with the serum levels and it was considered likely that these resulted from a "leak" rather than from local production of antigen. This report has been confirmed by many laboratories.

Amniotic fluid and breast milk. Hepatitis B surface antigen has been reported occasionally in amniotic fluid. This antigen has also been found in breast milk from antigen-positive mothers. A study in Taiwan of 147 babies born to mothers known to be surface antigen carriers did not reveal evidence of a relationship between breast-feeding and subsequent development of antigenaemia in the babies. The frequency of acquisition of antigen and surface antibody was almost identical among breast-fed and artificially fed infants in an area where vertical transmission of hepatitis B seems to account for 40–50% of carriers in Taiwan (Beasley *et al.*, 1975).

Bile. There are several reports of the finding of hepatitis B surface antigen in gall bladder bile of patients with antigenaemia but not in bile collected from the duodenum. The titre of antigen in gall bladder bile was approximately that in the serum. One group of investigators consider that the failure to detect the surface antigen in duodenal bile (and faeces) is due to the combination of the effect of dilution and the action of an intestinal inhibitor.

Faeces. Although hepatitis B surface antigen has been found in faeces and faecal extracts by several laboratories, this finding has not been confirmed. Insensitive techniques such as immunodiffusion and counter-immuno-electrophoresis were used and it is possible that the presence of several precipitin lines resulted in difficulties in interpretation. Another explanation for the discrepancy concerns the identification of an inhibitor of hepatitis B surface antigen in human faeces and in the intestinal mucosa. This inhibitor does not appear to be an antibody nor an enzyme but seems to be similar to the mouse intestinal factor which inactivates mouse hepatitis virus 3 (Molinari *et al.*, 1975). Another suggestion is that the presence of bacteria, such as *Pseudomonas aeruginosa*, in faeces, contaminated water and so on may prevent the detection of hepatitis B surface antigen by serological techniques (Weng *et al.*, 1975).

Urine. As in the case of faeces there are a number of reports on the detection of hepatitis B surface antigen in urine. Most of the results were

based on insensitive assay techniques and after considerable concentration of the samples. The antigen was detected more recently by radioimmunoassay in the first-morning specimen of urine, after 100-fold concentration, in 7 out of 43 carriers (Irwin *et al.*, 1975).

Incubation period
In the past, hepatitis type A and hepatitis type B were distinguished by their period of incubation, 20–40 days for hepatitis A and 60–180 days for hepatitis B. However, many recent studies in experimentally infected volunteers, recipients of blood and blood products, and others have revealed a much wider range of incubation periods for hepatitis type B, overlapping with that of hepatitis type A and extending up to 180 days.

Incidence
The trend in many geographical regions is towards increase in the incidence of type B infection. The precise reasons are difficult to evaluate but the following are important contributory factors. There is an ever-increasing demand for the transfusion of blood and blood products with improvement in medical care and with the increasing complexity of medical, surgical and dental procedures. Commercial blood bank practices are widespread in some countries. Mass immunization campaigns may be associated with an increase in syringe-transmitted infection especially if individual disposable syringes and needles are not used. The practice of tattooing, scarification, ear piercing and the use of inadequately sterilized instruments for other procedures such as acupuncture also increase the risk of infection. The widespread problem of narcotic addiction and parenteral drug abuse, the establishment of various cults (e.g. hippies) and the increasing mobility and migration of young adults to the large metropolitan centres must all contribute to the growing incidence of type B infection. There is now also considerable epidemiological and experimental evidence that hepatitis B is transmissible by non-parenteral means.

Age incidence
Hepatitis B is uncommon in children in Western communities except under certain circumstances such as children with Down's syndrome in institutions, multiply transfused children and children with defects in their immune response. The prevalence of hepatitis B surface antigen increases markedly in males in the age group of 15–34 years, and there is a general increase in the incidence of type B hepatitis among young adults. This relatively high incidence tends to decrease after early middle age. The epidemiological situation differs in the tropical and developing countries where a significant proportion of clinically well children carry

the surface antigen in their serum or have evidence of previous exposure to hepatitis B virus in the form of surface antibody and core antibody.

Modes of transmission

Hepatitis B virus may be transmitted by the transfusion of blood and certain blood components and by any procedure in which the skin or mucosa is penetrated by inadequately sterilized syringes, needles, instruments or implements, which have become contaminated with the blood or tissue fluid of a patient or a carrier. Transmission of hepatitis B infection by blood-sucking insects and by contaminated instruments used for scarification, tattooing, ear and nose piercing, acupuncture and ritual operations such as circumcision and blood letting are of importance. Spread by non-parenteral routes associated with poor socio-economic conditions and spread of the infection by the sexual route are new and important facets of the epidemiology of hepatitis B.

Transmission by transfusion of blood and blood components. Hepatitis B, and at least in some areas of the world the recently identified non-A : non-B form of hepatitis (Chapter 18), constitutes the main hazard of the transfusion of blood and certain plasma derivatives. Blood from hepatitis B surface antigen-positive donors carries a high risk of hepatitis B for the recipient (reviewed by Zuckerman, 1970, 1975a; WHO Reports, 1973, 1975, 1977). Marked variations in the prevalence of hepatitis B surface antigen in asymptomatic blood donor carriers (Chapter 13) have been found in different parts of the world. Prevalence also varies with such factors as socio-economic status, sex and age of the donor, the use of volunteer or paid donors and whether the donor lives privately or in an institution (WHO Report, 1973). The selection of blood donors and the sensitivity and specificity of the techniques used for screening blood donors for evidence of hepatitis B (Chapters 8, 9 and 10) clearly influence transfusion-associated transmission (reviewed by Zuckerman, 1975a; WHO Reports, 1973, 1975).

Blood derivatives were classified in the past according to the risk of hepatitis to the recipient (Zuckerman, 1975a). Whole fresh blood and single donor plasma were regarded as "average-risk" material; pooled plasma, fibrinogen and antihaemophilic factors were considered "high-risk" products. However, hepatitis B surface antigen has been found in all plasma components prepared by Cohn fractionation (WHO Reports, 1973, 1975). A sharp decrease followed in the prevalence of surface antigen in all plasma components by the screening of blood donors for this antigen, but in order to reduce the risk of contamination further it is recommended that *each unit* of plasma to be used for fractionation should

be tested for surface antigen by the most sensitive techniques available. Nevertheless, it must be stressed that fibrinogen, antihaemophilic factor (Factor VIII) and Factor IX remain "high-risk" hepatitis products particularly in view of the risk of contamination with the non-A : non-B virus(es).

Sexual transmission of hepatitis B. Higher rates of infection with hepatitis B virus are observed in the families of persistent carriers than in the rest of the community. Mouth-to-mouth transmission may be important. Hepatitis B surface antigen has been detected in the saliva of patients with acute hepatitis and in chronic carriers, and preliminary studies suggest that such saliva (and semen) is infectious as shown by intravenous inoculation of gibbons and chimpanzees. Since the surface antigen has also been detected in menstrual blood, vaginal secretions and semen, it is possible that the virus can cross mucosal surfaces exposed to these fluids during intercourse, and venereal transmission is thus a plausible mechanism. Over 30 years ago, during the early years of the Second World War, cases of acute hepatitis were recorded among wives of servicemen who received yellow fever vaccine contaminated with hepatitis B virus. In addition, two cases of hepatitis in husbands were recorded in 1946 subsequent to infection of women with rheumatoid arthritis who participated in volunteer studies on transmission of viral hepatitis. Recent studies have yielded a wealth of good circumstantial evidence for sexual transmission of hepatitis. More recently it was reported that as many as 18% of the spouses of patients with acute hepatitis B contracted the infection, but not any of their other close family contacts. In another study it seemed that sexual contact or close domestic contact was the most likely source of hepatitis B infection in 40% of 67 patients investigated. In many surveys a high prevalence of hepatitis B surface antigen and surface antibody has been found in patients attending venereal disease clinics, and promiscuity and particularly homosexuality emerged as factors of special importance. Szmuness *et al.* (1975) evaluated two separate aspects of the role of sexual behaviour in the spread of hepatitis B among over 2000 people in New York: firstly the possibility that this infection can be transmitted by vaginal intercourse and therefore that hepatitis B may be regarded as a venereal disease; and secondly, the indirect impact of certain sexual practices on the spread of hepatitis B virus in different population groups. Serological evidence of infection was sought by very sensitive laboratory techniques. A significant excess of hepatitis B infection was found in two promiscuous groups: patients with venereal diseases and their unrelated sexual contacts (15–18%); and male, but not female, homosexuals (37–51%). The prevalence of antigen among male homosexuals was 13 times higher than in the

controls, and the rate of surface antibody was four to five times more frequent than in the controls. Spouses of asymptomatic carriers of the antigen had a higher prevalence (26–28%) of infection, which was two to three times as frequent among spouses of the controls. Serological evidence of infection with hepatitis B virus was found more frequently in individuals who had a higher-than-average probability of exposure to potentially infective partners or whose sexual behaviour made such exposure more likely. For example, large numbers of sexual partners, long duration of homosexuality and predominantly rectal intercourse. Although the size of the lesbian sample was small, it is of interest that antigen carriers were not detected, although antibody was found among the lesbians with the same frequency as in heterosexual women. This low prevalence may be related to the small number of sexual partners that most lesbians have; in the homosexual sample surveyed the mean number of partners during the preceding 6 months was 1·8 for women compared with 20 for men.

An unexpected finding reported by Szmuness et al. (1975) was the high prevalence of hepatitis B in homosexuals whose sexual behaviour included rectal intercourse, and a relatively low prevalence in homosexuals with practices beyond the bounds of the ano-genital system. The explanation for this difference may lie in the reported high incidence of recurrent genital and peri-anal skin and mucosal sores in the former group.

It is extraordinarily difficult to prove by means of a cross-sectional retrospective study that hepatitis B can be transmitted by the venereal route in addition to the many other potential modes of transmission by the parenteral and inapparent parenteral routes. Not only is the collection of reliable information on sexual behaviour difficult, but spouses and high-promiscuity groups differ from each other in many respects which cannot be overcome by statistical methods. Nevertheless, several studies have now confirmed that in certain groups promiscuity, intimate contact with large numbers of sexual partners and extravaginal intercourse may be responsible for a substantial proportion of clinically apparent or inapparent infections with hepatitis B virus. But it could not be ascertained whether type B hepatitis is really transmitted by vaginal intercourse. Conn (1976) pointed out that "it must be borne in mind that people who sleep together may do other things as well. They may share eating utensils, razors, toothbrushes, skin abrasions, needles, lovers and bed bugs; and any of these or others could permit the intimate, but non-venereal transmission of viral hepatitis".

Transmission by mosquitoes and other blood-sucking insects. Although the modes of transmission of hepatitis B in hot climates are similar to those in other parts of the world, additional factors may be of importance, particularly repeated biting by blood-sucking arthropod vectors, traditional tattooing

and scarification and ritual circumcision. Preliminary results of investigations into the role which biting insects may play in the spread of hepatitis B are conflicting. No consistent association could be found between the notification of hepatitis and the months of high or low rainfall or with the period immediately after the rains in eight countries in Africa and Latin America. Although this does not exclude the possibility that biting insects may play a role in the spread of hepatitis, these observations imply that those mosquitoes which multiply with the rains may not be involved. In a study in New Guinea the incidence of hepatitis B surface antigen was compared, in the same population, with that of antibody to several arboviruses of groups A and B. The incidence of arbovirus antibodies correlated strongly with that of mosquito activity and it was inversely related to altitude. On the other hand, the incidence of detectable hepatitis B surface antigen was not related to either mosquito activity or to altitude. The prevalence of arbovirus antibodies in the population also increased cumulatively with age, whereas the incidence of hepatitis B surface antigen did not increase significantly with age. Moreover, the different prevalence rates of antigen among ethnic groups living in the same geographical areas are inconsistent with vector-borne infection. In studies of mosquitoes caught in the wild in East Africa, 15% of 187 pools of mosquitoes, representing eight different species, were found positive for hepatitis B surface antigen when tested by radioimmunoassay. The surface antigen was also found in 17% of 69 pools of mosquitoes from four different genera in West Africa when tested by the same technique, and Wills et al. (1976) calculated, in another study, that the minimum field infection rate for three species of mosquitoes was approximately one in every 200 mosquitoes.

Mosquitoes of the species Aedes aegypti were also fed experimentally on a persistent carrier of hepatitis B surface antigen, and the rate of disappearance of detectable antigen was tested in the mosquitoes, which were subsequently maintained at 30°C. The antigen disappeared in parallel with blood meal digestion. In other similar studies the surface antigen was detected for up to 6-7 days after feeding.

In a laboratory study, seven species of mosquitoes were fed artificially on blood containing hepatitis B surface antigen. Radioimmunoassay showed that all the ingested antigen disappeared in parallel with digestion of the blood meal by the mosquito. There was no evidence of replication of hepatitis B antigen. These results indicated that the species of mosquitoes examined do not serve as cyclo-propagative vectors of hepatitis B. Nevertheless, mechanical transmission by blood-sucking arthropods is a possibility for hepatitis B, as for many other diseases. Evidence has

recently been obtained showing multiple feeding by mosquitoes during a single feeding cycle, usually after the insect has been disturbed. Blood group serology revealed the presence of erythrocytes from different individuals in the gut of a significant proportion of fed mosquitoes. These observations may be taken to imply that it may also be possible for hepatitis B to be transmitted mechanically by mosquitoes from person to person, but further work is required to confirm this hypothesis.

Bed bugs live more intimately with humans than do mosquitoes, take blood meals and could transfer blood and hepatitis B virus from one occupant of a bed to another. Hepatitis B surface antigen was detected in one (5·6%) out of 18 pools of engorged bed bugs of the species *Cimex hemipterus*. In a laboratory study two species of bed bugs were artificially fed on blood from a patient with acute hepatitis B. Hepatitis B surface antigen remained detectable in the bed bugs for over 4 weeks, and juvenile bed bugs contained antigen after moulting, at a time when the bug usually starts to search for a host and will refeed. In the course of another investigation, carried out in Senegal by Wills *et al.* (1977), bed bugs were collected on four separate occasions from the bedding in the huts of village dwellers. All the bed bugs were identified as *C. hemipterus*, the predominant species in West Africa and other tropical areas. Hepatitis B surface antigen was detected in unengorged nymph and adult bed bugs in each of the first three collections. Three out of 28 specimens were antigen-positive in the first collection and three out of 17 specimens were positive in the second collection. In the third collection, six out of nine bed bugs were positive when the bed occupant was known to be hepatitis B surface antigen-positive. The antigen was also detected in three out of 89 bed bugs in the fourth collection kept alive without a blood meal for 30 days. Of particular interest was the finding of the *e* antigen, which is a marker of infectivity of hepatitis B virus, in one engorged and one unengorged bed bug. The above results are the highest field infection rates with markers of hepatitis B virus reported so far in any insect species. Clearly bed bugs feeding on the occupants of the same bed could amplify the risk of hepatitis B infection. There is undoubtedly a need to examine closely and objectively these and other blood-sucking insects, and the entire matter deserves further epidemiological and experimental investigation.

Other modes of transmission of hepatitis B. The association between tattooing and hepatitis B has been recognized for many years and there are numerous reports of outbreaks in the literature. Cases of hepatitis B have also been reported in association with ear piercing, acupuncture, possible transmission by human bite, exudate from decubitus and other sores, and splashing of blood or serum on mucosal surfaces including the con-

junctiva. These reports do not, by any means, comprise all the known modes of transmission of hepatitis B infection but they do serve to emphasize some of the variegated means of spread of this virus, which is well illustrated by the epidemics of hepatitis from 1957–1966 amongst Swedish track-finders involving some 600 cases. A number of interesting and some speculative explanations were put forward. It was suggested, for example, that twigs, thorny bushes or barbed wire which became contaminated by the blood of a carrier transmit the infection to other runners who sustain small scratches and cuts on the same thorns. However, it seems more likely that actual infection occurred from water used for bathing or by using the same towels. It was clear that the infection was type B viral hepatitis and this suggestion was supported by finding hepatitis B surface antigen in 32 (46%) out of 69 patients whose sera had been stored since the outbreaks in 1961–1962. The antigen was found in all the sera obtained within the first 9 days of the onset of symptoms.

Clustering of hepatitis B in families
It is clear from the above account that there are different possible ways of transmission of hepatitis type B from person to person including spread by parenteral, inapparent parenteral and non-percutaneous routes. Numerous surveys in different parts of the world have revealed evidence of clustering of this infection within family groups. On the whole, the frequency of serological evidence of hepatitis B infection in family contacts does not reflect maternal and venereal transmission and an antigen-carrier blood donor may not necessarily be the primary or "index" case in these families. In some studies in high-prevalence groups it was found that the proportion of positive relatives was more or less similar in parents, siblings and offspring, which does not accord with the hypothesis of autosomal recessive inheritance of hepatitis B surface antigen. There is a striking relationship between increasing age and serological evidence of infection with hepatitis B virus, and a dramatic association with socio-economic status—the highest prevalence of infection being in the poor socio-economic groups. It seems likely that most individuals within a family acquired their infection, at least in part, by non-parenteral (or inapparent parenteral) means. The nature of this route is not known, but the antibody acquisition patterns do not support oral or venereal spread as the principal mode of non-parenteral infection. Perhaps intimate and prolonged contact is necessary. The infection with hepatitis B virus in these population groups seemed to be mainly subclinical, in contrast to hepatitis after parenteral exposure to blood or narcotic drugs in which the ratio of subclinical to clinical infection appears to be 3 : 1.

4 | Pathology of the liver in acute viral hepatitis

A number of factors make it difficult to describe the evolution of morpho-logical changes in the liver in a disease which varies in its severity from an asymptomatic or anicteric infection to massive necrosis of the liver. Thus, biopsy of the liver is not required during the incubation period of the infection in anicteric patients or in patients with the typical mild illness. On the other hand, biopsy should not be carried out in severe liver damage or in patients with disturbed blood clotting. Some generalizations are therefore inevitable in describing the structural changes in the liver during the course of viral infection and little is known of the earliest events in human infection, although there is more information on the histological changes in the liver of experimentally infected chimpanzees (Dienstag *et al.*, 1976).

Two features are constant in acute human viral hepatitis: parenchymal cell necrosis and histiocytic periportal inflammation. In general the reticulin framework of the liver is well preserved except in some cases of massive and submassive necrosis. The liver cells show various forms of necrotic changes, which are usually multifocal, but necrosis frequently tends to be zonal with the most severe changes in the centrilobular areas. Individual hepatocytes are commonly swollen and may show ballooning, but they may shrink. Swollen cells typically show a granular "ground glass" appearance of the cytoplasm. Shrunken cells give rise to acidophilic bodies. Dead or dying rounded liver cells are extruded into the peri-

sinusoidal space. The variations in the size of the nuclei and in their staining quality is useful in diagnosis. Fatty changes in the liver are conspicuous by their absence.

A mononuclear cellular infiltration, which is particularly marked in the portal zones, is the characteristic mesenchymal reaction. This is accompanied by some bile ductular proliferation. In some cases during the early phases of the illness polymorphonuclear leucocytes and eosinophils may be prominent. The mononuclear inflammatory changes are scattered throughout the sinusoids and in all parts of the lobules involved in focal necrosis. The lost hepatocytes are replaced mainly by mononuclear cells. The Kupffer cells and endothelial cells proliferate and they are enlarged, the Kupffer cells often containing excess lipofuscin pigment. During the icteric phase of the average case of hepatitis the wall of the tributaries of the hepatic vein may be thickened, frequently infiltrated, and the proliferation of the lining cells in the terminal hepatic veins would justify the term "endophlebitis". Cholestasis may occur in the early stages of viral hepatitis and plugs of bile thrombi may be seen in the bile canaliculi. Occasionally cholestatic features dominate the picture but spotty necrosis is almost invariable.

Spotty or focal necrosis with the associated mesenchymal reaction may also be found in anicteric hepatitis, although on the whole the lesions tend to be less severe. At the other end of the spectrum, in fulminant hepatitis, there is rapid massive necrosis of the liver cells. At a later stage of the evolution of the lesion of acute hepatitis there is often a variable degree of collapse and condensation of reticulin fibres and accumulation of ceroid pigment and stainable iron in large phagocytic cells, first within the lobules and later also in the portal tracts.

Repair of the liver lobule occurs by regeneration of the hepatocytes. Frequent mitoses, polyploidy, atypical cells and binucleated cells are found. There is a gradual disappearance of the mononuclear cells from the portal tracts, but elongated histiocytes and fibroblasts may remain.

Morphologically, the outcome of acute viral hepatitis may be complete resolution, massive necrosis, chronic persistent or aggressive hepatitis, resolution with scarring or cirrhosis. Cirrhosis may result from extensive confluent necrosis leading to septum formation, architectural distortion and nodular hyperplasia, or it may follow chronic aggressive hepatitis, or both processes may occur together. Chronic liver disease following acute viral hepatitis may thus be the result of necrosis, collapse of the reticulin framework, the formation of scars and nodular hyperplasia. Other factors

may include immunological processes and viral persistence in the liver.

It is generally agreed that in humans it is not possible to distinguish between the changes in the liver in hepatitis A and B on morphological grounds. Furthermore, it is impossible to distinguish histologically between viral hepatitis and hepatitis induced by a number of drugs. Some drugs induce hepatitis which closely simulates viral hepatitis, whereas other drugs may produce a picture which mimics the cholestatic form of viral hepatitis.

The pattern of histological changes in the liver is also similar in hepatitis types A and B (Dienstag *et al.*, 1976) and consists of conspicuous focal activation of sinusoidal lining cells; accumulations of lymphocytes and histiocytes within the parenchyma, often replacing hepatocytes lost by cytolytic necrosis; mild diffuse hepatocytic changes with occasional coagulative necrosis in the form of acidophilic bodies; and focal regeneration and portal inflammatory reaction with alteration of bile ductules. However, the lesions in hepatitis A develop earlier and the duration of morphological changes is shorter, while the lesions in hepatitis B linger on, fluctuate and regress slowly. There is also a difference in distribution of the lesions. In hepatitis A, the localization of parenchymal changes is predominantly periportal, whereas in hepatitis B the lesions are diffuse and, if anything, accentuated around the hepatic vein tributaries, and streaks of focal necrosis may extend from portal tracts to hepatic vein tributaries. The portal inflammatory reaction in hepatitis A is more severe than the parenchymal lesion. It should be noted that the main difference between hepatitis A and hepatitis B infection in chimpanzees and human hepatitis lies in the relative paucity of hepatic necrosis compared to the marked portal inflammatory reaction.

ULTRASTRUCTURAL CHANGES IN THE LIVER IN VIRAL HEPATITIS

The electron microscope has been used extensively for the examination of the fine structure of the liver biopsy material obtained from patients with viral hepatitis and in the search for virus particles. Most of the changes in fine structure appear to be non-specific and tend to be very variable, ranging from minor alterations to total necrosis.

The first response of the hepatocyte to injury is an alteration in the profile of the rough endoplasmic reticulum. The rough endoplasmic reticulum becomes dilated and disrupted and the ribosomes, which

usually line the membranes, become detached. The degree to which this dilation occurs varies from cell to cell and the swelling may be associated with the formation of small vesicles as a result of disruption. When the swelling is extreme it is responsible for the appearance of the "balloon cell" which is readily recognized in the light microscope. These changes in the rough endoplasmic reticulum are a non-specific reaction to injury. The smooth endoplasmic reticulum is also affected and hypertrophy occurs. In some liver cells aggregates of smooth membranes occupy large areas of the cytoplasm and these may take the form of whorls, which under the light microscope are seen as eosinophilic bodies. These eosinophilic bodies may be a form of focal cytoplasmic degradation of the cell organelles. The number and size of lysosomes are increased in hepatitis. In some cells the lysosomes are large and represent autophagic vacuoles or cytoplasmic degradation. Primary mitochondrial changes, particularly swelling, are usual in any type of advanced cell damage and are particularly conspicuous in the "balloon cells". Loss of the outer membranes of mitochondria may also be observed in the early stages of the disease. The cytoplasm of occasional cells may be packed with mitochondria. In contrast to the cytoplasmic changes, nuclear abnormalities are not usually marked. Pyknosis is occasionally seen. Nuclear inclusions resulting from cytoplasmic invaginations are sometimes found.

The microvilli on the sinusoidal surface of the liver cells are decreased in number and those present are frequently oedematous. The space of Disse is widened and is filled with debris and collagen fibrils. Discontinuous basement membranes are usually visible beneath the endothelial lining. The changes in the secretory apparatus of bile are related to the degree of cholestasis. Aggregates of bile may be found in normal hepatocytes as well as in ballooned cells. Bile thrombi are found in the canaliculi and the cell membranes in contact with the thrombi are usually damaged. The changes in the fine structure of the biliary epithelium and the bile canaliculi are not specific to viral hepatitis. The Kupffer cells are increased in size and number. The hypertrophy is due to swelling of cytoplasm with extensive vacuole formation. Some of the vacuoles contain fragments of hepatocytes or acidophilic bodies, whereas others contain glycogen or lipofuscin. Other cells implicated in the inflammatory response are found. The endothelial layer is often multilayered in areas of hepatocellular injury.

More recently, Schaffner et al. (1977) reported the results of examination by electron microscopy of coded liver biopsy specimens from chimpanzees

infected experimentally with hepatitis A or B virus. The hepatocytes from chimpanzees with hepatitis A differed ultrastructurally from those with hepatitis B and both differed from hepatocytes in human hepatitis. Hepatitis A was characterized by unidentified, large, dense and irregular heterochromatin-like particles in the nuclei of hepatocytes coincidental with peak aminotransferase activity. These particles differed from the 27-nm virus particles of other studies detected by electron microscopy in the cytoplasmic vesicles of the hepatocytes of experimentally infected marmosets and chimpanzees. In addition, during the peak of the illness, mitochondrial cristae were curled and had a coarse matrix and clusters of smooth endoplasmic reticulum were tightly packed. In contrast, hepatitis B was recognized by the presence of hepatitis B core particles in the nuclei and the cytoplasm of the hepatocytes showed mainly hypertrophy of tubular endoplasmic reticulum. The mitochondria were normal in appearance.

INTRACELLULAR VIRUS PARTICLES IN HEPATITIS A

The CR 326 strain of hepatitis A virus was found in ultra-thin sections of the livers of infected *Saguinus mystax* marmosets (Hilleman *et al.*, 1975). The particles were 27 nm in diameter and were found in the cytoplasm but not in the nucleus. Occasionally the particles were localized in vesicles of various sizes. Schulman *et al.* (1976) also reported the finding by thin-section electron microscopy of 27-nm virus-like particles in the cytoplasm of hepatocytes and inside vesicles in liver biopsies of chimpanzees obtained 24 days after inoculation. Subsequently hepatitis A virus antigen was demonstrated by immunofluorescence in liver biopsies from infected chimpanzees. The antigen was detected by immunofluorescence before the shedding of virus in the faeces, and before elevation of serum alanine aminotransferase or histological changes in the liver. In the early positive biopsies the antigen was distributed diffusely in the cytoplasm of many cells in the liver, but later there was only focal distribution of the antigen in the cytoplasm of a few hepatocytes and Kupffer cells.

INTRACELLULAR VIRUS PARTICLES IN HEPATITIS B

Characteristic hepatocytes with eosinophilic "ground glass" cytoplasm are usually found in liver biopsies of persistent carriers of hepatitis B. Many

cells contain an increased amount of brown pigment, presumably lipo-fuscin or clumped basophilic material near their biliary pole. Usually they are rich in glycogen and free of iron. The nuclei appear to be normal, but are often double. The "ground glass" hepatocytes are scattered or arranged in cords or clusters without predilection for any part of the liver lobule. These hepatocytes are always associated with many hepatocytes with bright hepatitis B surface antigen specific cytoplasmic fluorescence. In serial sections examined by both light microscopy and immuno-fluorescence the "ground glass" hepatocytes correspond to the cells with cytoplasmic antigen. Examination by electron microscopy of specimens containing many "ground glass" hepatocytes has shown 20–30-nm spherical particles and long filamentous forms in the cisternae of the endoplasmic reticulum, and in many such hepatocytes the endoplasmic reticulum is in excess. The surface antigen has also been demonstrated in the cytoplasm of hepatocytes by staining with orcein and aldehyde-fuchsin and more recently by immunoperoxidase (e.g. Sachdeva *et al.*, 1976; Afroudakis *et al.*, 1976; Lamothe *et al.*, 1976; Portmann *et al.*, 1976; Busachi *et al.*, 1978).

Hepatitis B core antigen has been localized in the nuclei of hepatocytes both in the particulate form measuring 21–25 nm in diameter, by immune electron microscopy using a ferritin label, and by immuno-fluorescence and immunoperoxidase techniques (see, for example, Ray *et al.*, 1976; Lamothe *et al.*, 1976).

Trepo *et al.* (1976) reported the detection of hepatitis B *e* antigen by immunofluorescence in the cytoplasm of hepatocytes of carriers of the surface antigen. These findings were not confirmed by Arnold *et al.* (1977) who localized the *e* antigen in the nuclei of hepatocytes by a double staining immunofluorescence procedure as well as by blocking techniques. Core antigen and *e* antigen were found very frequently simultaneously in the same nuclei, although in some cells only the core antigen was detected and in others only *e* antigen. The precise localization of the *e* antigen thus remains to be determined.

5 | The clinical features of viral hepatitis

The clinical picture of viral hepatitis varies in its presentation from inapparent or subclinical infection, slight malaise, mild gastro-intestinal symptoms and the anicteric form of the disease, to acute icteric illness, severe prolonged jaundice and chronic liver disease. The incidence of individual symptoms and signs varies both in different epidemics and in sporadic cases.

There are no constant clinical differences to help distinguish between hepatitis A and hepatitis B during any phase of the illness. It is, nevertheless, generally accepted that there is an increased incidence of severe illness in patients with hepatitis B than in hepatitis A, but this may be related, in many cases, to other underlying factors such as a disease requiring transfusion. At the same time, it must be emphasized that acute viral hepatitis, in all its forms, is common, yet clinical evidence of involvement of the liver is infrequent and this is particularly true in the case of children, in whom the ratio of anicteric to icteric illness has been estimated to be as high as 10 or more to 1.

ACUTE ANICTERIC HEPATITIS

This form of hepatitis is characterized by malaise, anorexia and various gastro-intestinal disturbances. Fever may be present. In children these symptoms may be very mild. Clinically, the liver is enlarged and tender, bile is present in the urine and biochemical tests reveal elevation of serum alanine and aspartate aminotransferases and a transient rise in conjugated serum bilirubin. In children the infection may be almost asymptomatic and it may be recognized only on the basis of symptoms occurring during an epidemic of hepatitis or after known exposure to infection and on the results of biochemical tests. In general terms the clinical picture of acute icteric hepatitis follows an incubation period of 28–30 days in the case of hepatitis A and 40–180 days in the case of hepatitis B. The extent of histological changes in the liver is variable and the severity of the lesions may be quite unrelated to the mild symptomatology of the illness. The mild nature of the clinical manifestations does not preclude progression of the hepatic lesion to chronic liver damage. The true incidence of permanent liver damage following viral hepatitis type B and non-A : non-B infection is as yet undetermined with any degree of accuracy. However, it seems that chronic liver damage does not follow infection with hepatitis A virus.

ACUTE ICTERIC HEPATITIS

The duration of symptoms during the preicteric phase of the illness varies from 1–14 days, but it may be longer. The average is 5–10 days. Marked anorexia and nausea are prominent. Distaste to smoking is an unexplained observation, but it is not a constant feature. Headache, generalized myalgia, arthralgia, skin rashes and fever may be present in a number of patients. The predominant presenting symptoms, however, are gastro-intestinal and toxic features. The preicteric stage progresses to the icteric phase with the appearance of dark urine, clay-coloured stools and jaundice. Jaundice may be the presenting symptom in between 8 and 22% of patients. With the appearance of jaundice there is usually a rapid subjective improvement in symptoms. The jaundice usually deepens during the first few days and persists for 1 or 2 weeks. The faeces then darken again and the jaundice gradually diminishes, at first rapidly and then more slowly over a further period of two weeks or so. In children, the prodromal features may be mild or even absent, although when present

anorexia tends to be prominent. Generalized features such as myalgia and arthralgia are also uncommon in children and jaundice may last for only a few days. The posticteric or convalescent stage follows and complete recovery in the adult usually takes place within a few months. The post-icteric phase in children is short and recurrence of symptoms is rare. Recurrence of symptoms and indeed relapses with jaundice may occur and *sequelae* such as chronic hepatitis and posthepatic cirrhosis may follow.

OTHER FORMS OF VIRAL HEPATITIS AND *SEQUELAE*

Cholestatic hepatitis
This form of hepatitis usually starts with jaundice and it is characterized by a progressive intrahepatic obstructive type of jaundice. Pruritus is a conspicuous feature. Transient periods of cholestasis are not infrequent in viral hepatitis. However, the most common form of cholestatic hepatitis is characterized by unexplained persistence of jaundice accompanied by increasing serum bilirubin, cholesterol, lipid and serum alkaline phosphatase levels. Cholestatic hepatitis is more frequently observed at the end of a prolonged episode of hepatitis, but sometimes it may be an early presenting feature. Cholestasis is believed to be the result of mechanical and obstructive elements. Functional disturbance of the liver cells is associated with overload of bile in some cells, in the canaliculi and Kupffer cells. Fissures occur across the cytoplasm of the cells as a result of rupture of some cell membranes and bile stasis in canaliculi, and these establish shunts between the biliary system and the blood. Liver biopsy shows histological evidence of marked bile stasis and the morphological features of hepatitis. Cholestatic hepatitis is ultimately followed by a complete recovery and progression to cirrhosis does not occur.

Fulminant hepatitis
The term fulminant hepatitis describes a rare form of viral hepatitis which was referred to in the past as acute yellow atrophy of the liver. This form of the infection has a very high mortality and death often occurs within 10 days of onset as a result of massive necrosis of the liver. Progressive deep jaundice and widespread haemorrhages are a constant feature, but at times the course of the disease may be so rapid that jaundice may be inconspicuous.

Chronic persistent and chronic aggressive hepatitis

Remissions and recurdescences occur at intervals. In some patients the original illness is typical of acute anicteric or icteric hepatitis, but this is not necessarily so. Two main categories of chronic hepatitis have been proposed: chronic persistent hepatitis and chronic active (aggressive) hepatitis (Fogarty International Center, 1976).

Chronic persistent hepatitis is a benign, non-progressive disease characterized by chronic inflammatory infiltration, which is mostly portal in distribution. The lobular architecture is well preserved and there is little or no fibrosis. Piecemeal necrosis of the limiting plate is absent or minimal. The histological features of acute hepatitis may be super-imposed, but generally these are less pronounced. Clinically, the majority of patients have no complaints. Fatigue, weight loss or minor abdominal symptoms may be present. Occasionally the liver is tender or slightly enlarged.

In chronic active hepatitis there is chronic inflammatory cell infiltration involving the portal tracts and extending into the parenchyma, with moderate or severe piecemeal necrosis extending outward into the paren-chyma from the limiting plate and the formation of intralobular septa. The liver architecture is disturbed and there is no nodular regeneration. Changes similar to those present in acute hepatitis B may be present in the lobule together with the features of cholestasis. Fibrosis or cirrhosis, usually of the macronodular type, occurs in more advanced cases. Clinic-ally, females are more commonly affected. The onset may be insidious or apparently sudden. In milder cases the symptoms may be non-specific, but in more severe cases jaundice, ascites, hepatosplenomegaly and other features of parenchymal liver disease are observed. Systemic manifestations such as arthralgia, rash and fever are common and some patients have inflammatory disease of other organs.

Cirrhosis following hepatitis

This term is applied to a lesion, which besides prominent fibrosis, shows additional features, particularly parenchymal nodules. Cirrhosis develops when extensive and repeated parenchymal destruction is followed by fibrosis and by parenchymal regeneration resulting in regenerating nodules of various sizes. In addition to regenerating nodules, passive nodules form when septa dissect the lobular parenchyma so that no remnant of the original lobules is seen. The septa may be the result of bridging or lobular

collapse or of active fibroplasia. Cirrhosis is a terminal stage of chronic hepatitis induced by many different aetiological factors.

The problem of whether cirrhosis may follow acute viral hepatitis has been the subject of debate for many years. Large-scale follow-up investigations of many patients with viral hepatitis among young military personnel and others have yielded no definite evidence of cirrhosis which could be attributed to this infection. Other observers, however, concluded that cirrhosis may follow acute viral hepatitis although in the majority of the reports the evidence for this was, of necessity, largely circumstantial. However, if acute hepatitis does result in permanent disruption of the normal lobular architecture of the liver then cirrhosis is inevitable. The progression of viral hepatitis, even from the anicteric form, to cirrhosis has been documented in a number of studies where serial liver biopsies were obtained. The incidence of permanent liver damage after acute viral hepatitis was, however, believed to be very small. The development of specific and sensitive serological tests for hepatitis B antigens have altered previous concepts on the relationship between infection with type B viral hepatitis and progression to chronic active hepatitis, cirrhosis and possibly primary liver cell carcinoma.

6

The morphology of the hepatitis viruses

Electron microscopy and immune electron microscopy have played a major role in the rapid advances which have been made during the last decade in the complex field of human hepatitis. The technique of negative staining for high resolution electron microscopy utilizes the principle of surrounding within a rigid electron-dense material particles such as viruses, isolated subcellular components and other biological structures. There is usually very good preservation of the biological material under test with minimum distortion of structure. Negative staining results in high contrast and is relatively unaffected by impurities of small molecular size which merge with the background and conversely large-sized impurities are clearly discernible from virus particles. The main disadvantage of the negative staining technique is that although only relatively small amounts of the material to be tested are required, a high concentration of particle suspension is usually required, often 10^9-10^{12} particles ml^{-1}. The addition of serum containing specific antibody to the material under examination leads to the formation of antigen–antibody aggregates, a procedure which would concentrate particles within the immune complex thereby enhancing the sensitivity of this technique, so that in experienced hands a concentration of virus particles in the order of $10^4-10^{4.5}$ particles ml^{-1} may be visualized.

HEPATITIS B VIRUS

The first examination by electron microscopy of serum specimens containing the surface antigen was carried out on sucrose gradient fractions of sera collected from three different patients, one suffering from acute myelogenous leukaemia, another with chronic reticuloendotheliosis and the third patient with Down's syndrome and chronic hepatitis. Negative staining with sodium silicotungstate revealed spherical particles measuring 19–21 nm in diameter with surface knob-like "subunits" about 3 nm in diameter. Elongated particles varying in length from less than 50 nm to 230 nm were also noted. The particles were aggregated by the addition of specific rabbit gamma-globulin to the surface antigen. Subsequently, the ultrastructural features of virus-like particles seen by electron microscopy in immune aggregates of serum containing hepatitis B surface antigen were described in samples obtained from a proven long-term asymptomatic carrier of hepatitis B and from two heroin addicts who contracted acute hepatitis after sharing syringes. The main antigenic constituent was a pleomorphic roughly spherical particle measuring approximately 20 nm in diameter, but with a range of 16 to 25 nm. An outstanding feature was the presence of many tubular forms with a constant diameter close to 20 nm and often several hundred nanometres in length. Another notable finding was the very marked morphological heterogeneity of the particles which included many different shapes and forms. Although it was possible to resolve that the spherical particles were formed of subunits, it was not possible to interpret the surface structure. The tubular forms frequently displayed a regular transverse periodicity of approximately 3 nm on the surface. Many tubules displaying several right angles were also seen and this angularity appeared to be a unique feature of these structures. Bulbous or rounded swellings were frequently seen at either or both ends of the tubular forms. None of these particles were found in normal human sera reacted with hepatitis B antisera.

Yet another type of virus-like particle was soon described in the serum of patients with hepatitis type B. These particles measured about 42 nm in diameter and consisted of an inner body about 28 nm in diameter with a 2-nm shell and an outer coat about 7 nm in thickness. Several tadpole-like forms were found, with the tail resembling a typical tubular form in continuity with the outer coat of the large spheroidal particle. The large and small spherical particles and the tubules formed a mixed aggregate when reacted with hepatitis B antiserum indicating that they all
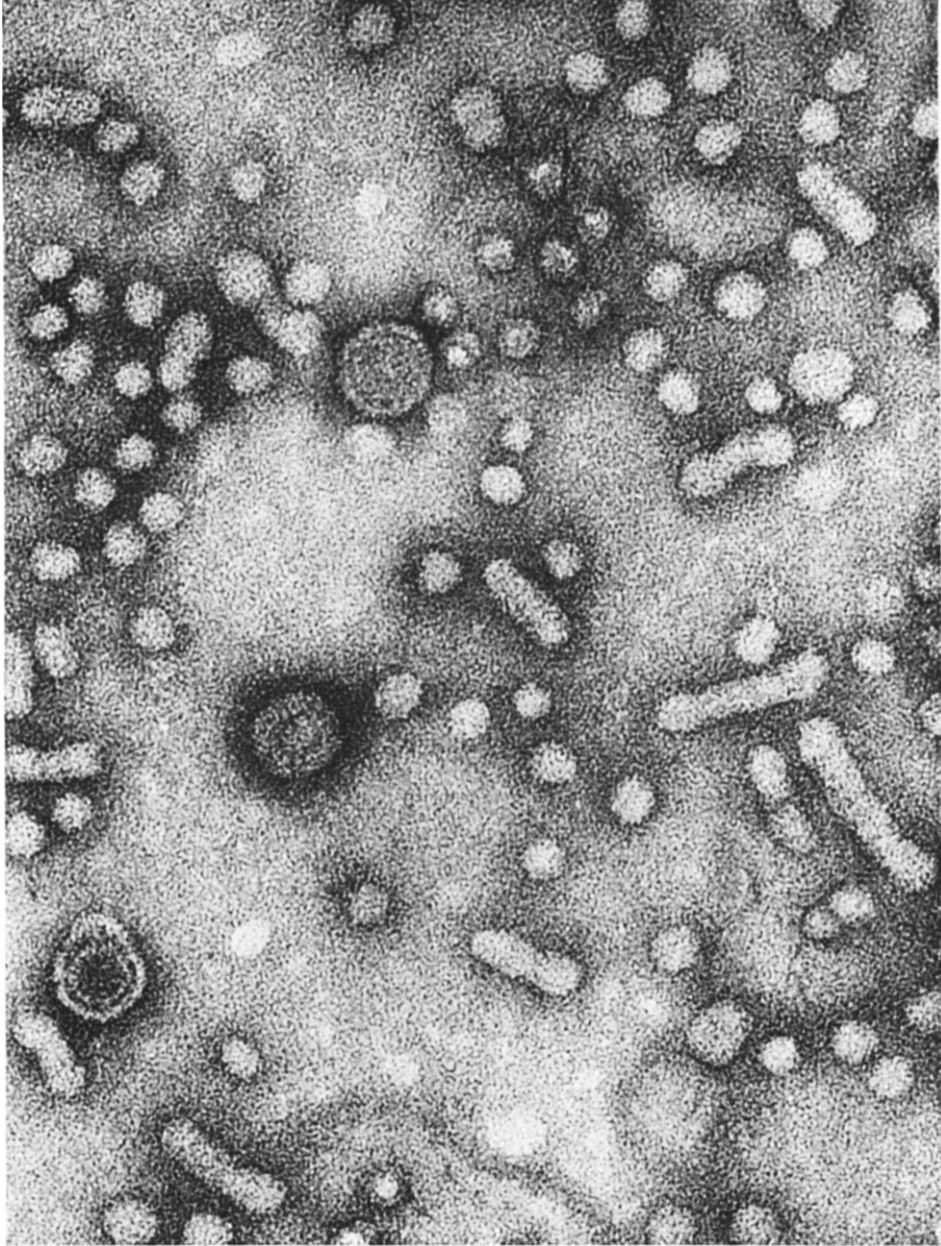
FIG. 6.1. Whole serum showing the three morphological forms of hepatitis B: small pleomorphic spherical surface antigen particles; tubular forms of varying length; and complete hepatitis B virions, double-shelled with varying degrees of stain penetration.
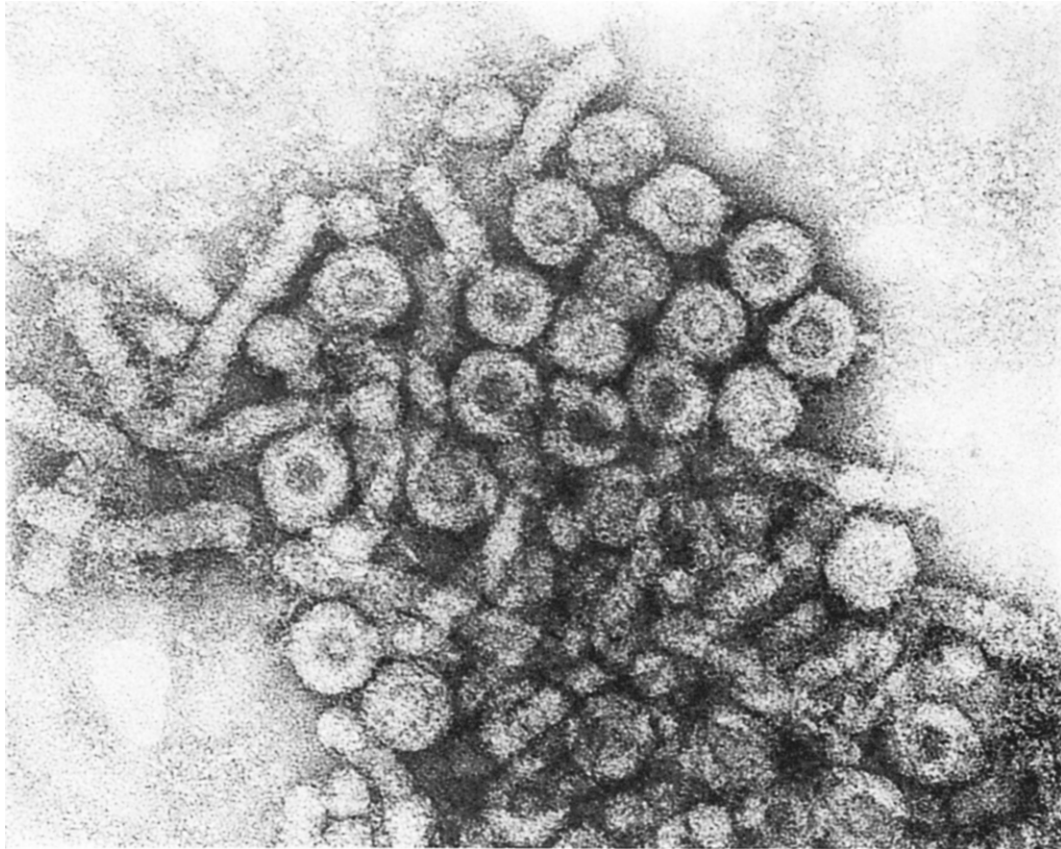× 252 000.

FIG. 6.2. An aggregate made up of many 42-nm virus particles, tubular forms and a few small spherical particles. × 252 000.

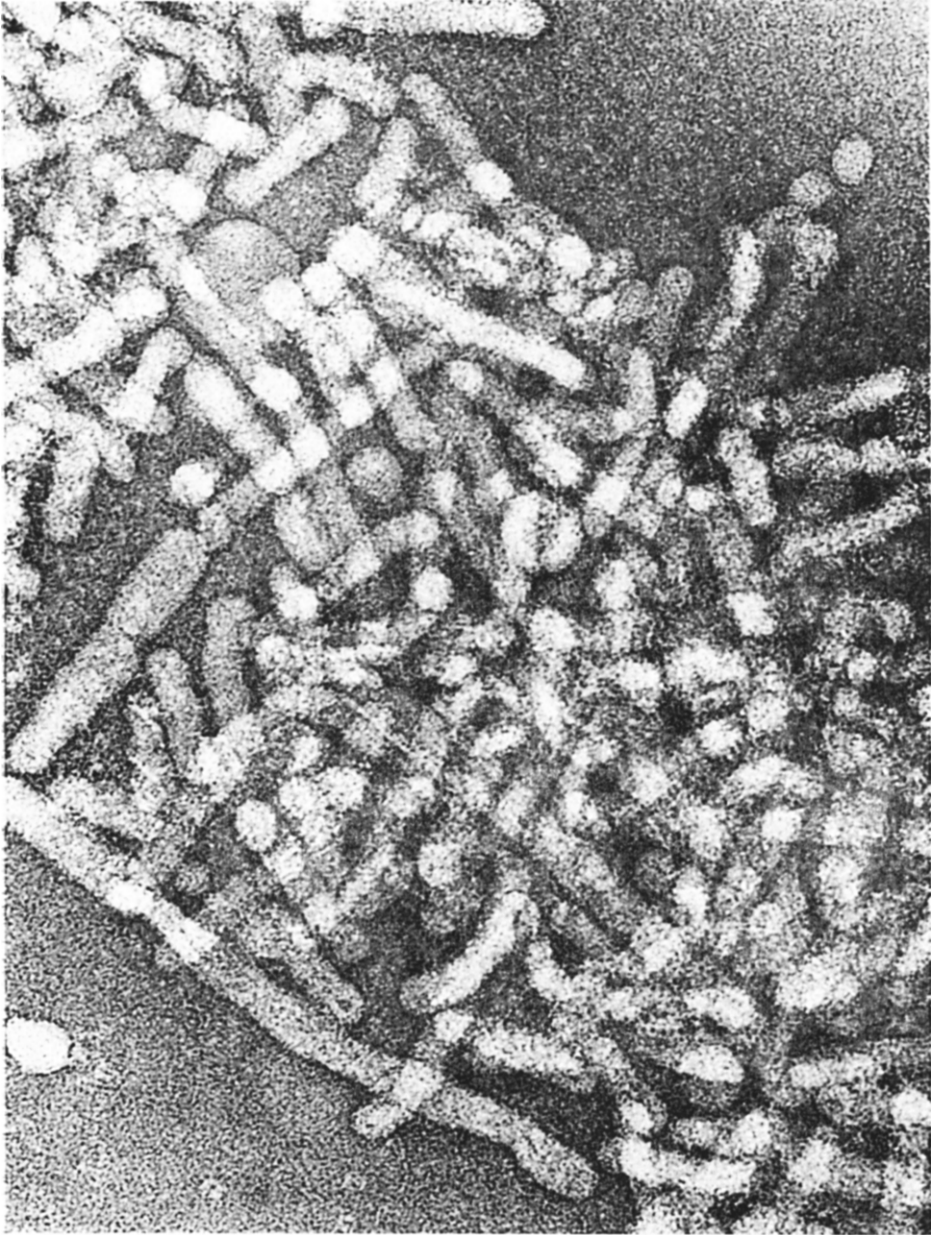FIG. 6.3. Electron micrograph of a serum from a hepatitis B carrier. Unusually large numbers of tubular forms of the surface antigen were found. × 252 000.
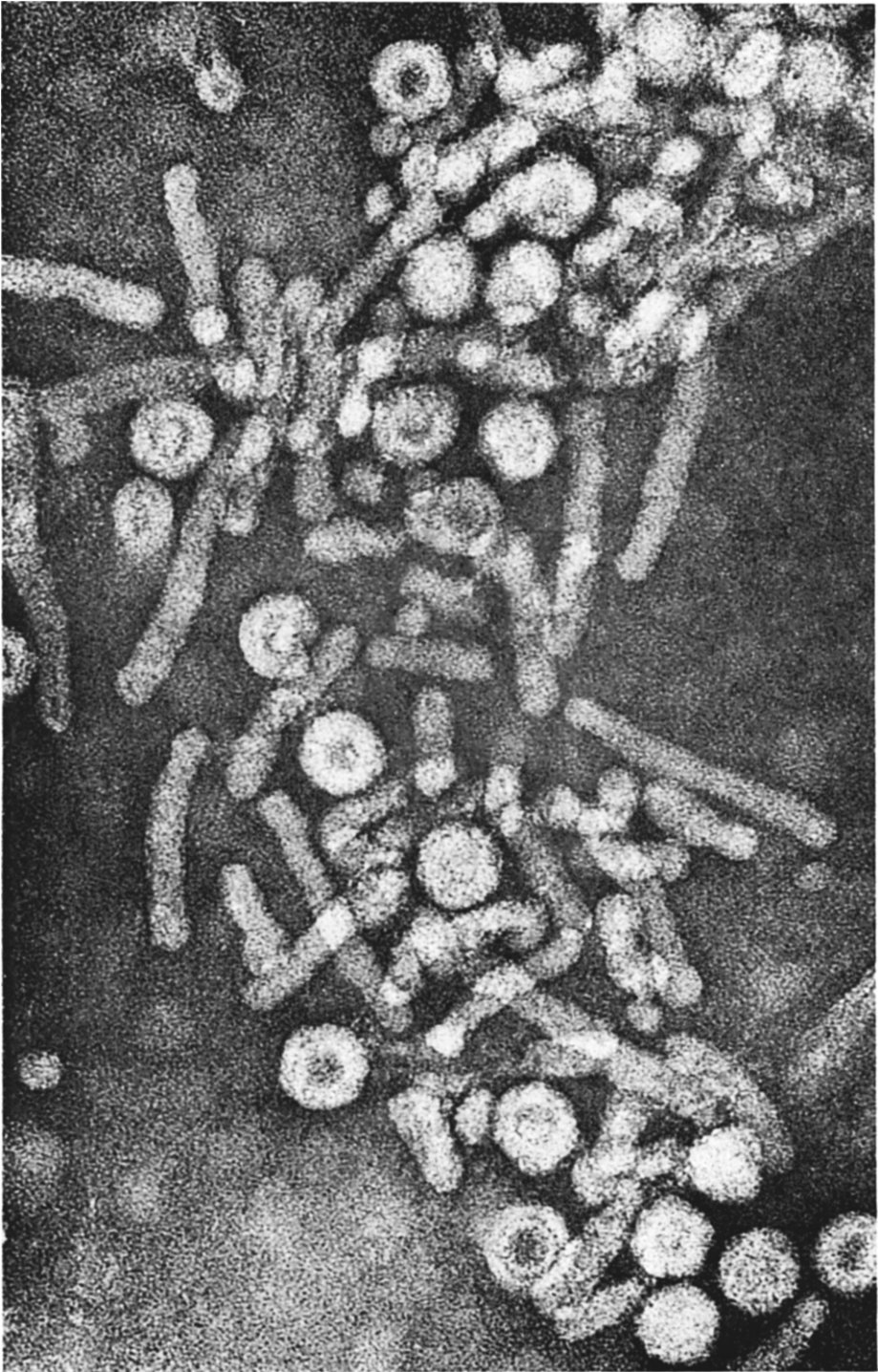
FIG. 6.4. Both solid and double-shelled forms of the hepatitis B virus are shown in this electron micrograph. × 252 000.
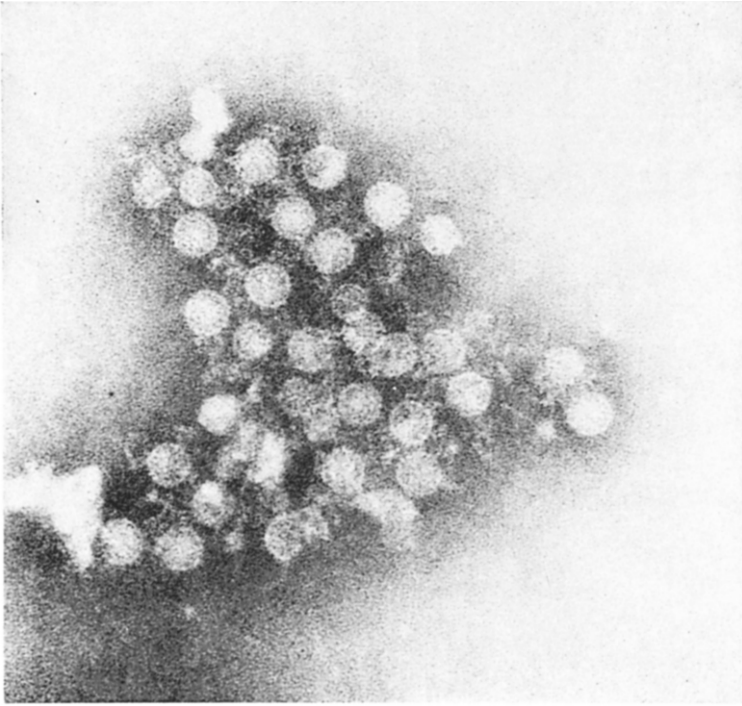
FIG. 6.5. Particles measuring 25-27 nm in diameter in a liver homogenate from a patient who died from acute hepatitis B. × 280 000. (Electron micrograph kindly provided by Dr June D. Almeida.)

shared a common surface antigen. It was suggested that the double-shelled 42-nm particles represent the virus of hepatitis B whereas the small particles and the tubular forms of the antigen are non-infectious surplus virus-coat protein (Figs 6.1–6.4).

Examination by electron microscopy of liver homogenates prepared from two patients who died of acute antigen-positive hepatitis revealed virus-like particles measuring 25–27 nm in diameter and displaying the morphology of many small enteroviruses (Fig. 6.5). Examination by transmission electron microscopy of ultra-thin sections of the liver obtained at necropsy from six patients who suffered from a variety of malignant lymphoproliferative disorders and in whose serum hepatitis B surface antigen was detected revealed the presence of 20-nm particles in the nuclei of the hepatocytes in the tissue from antigen-positive patients
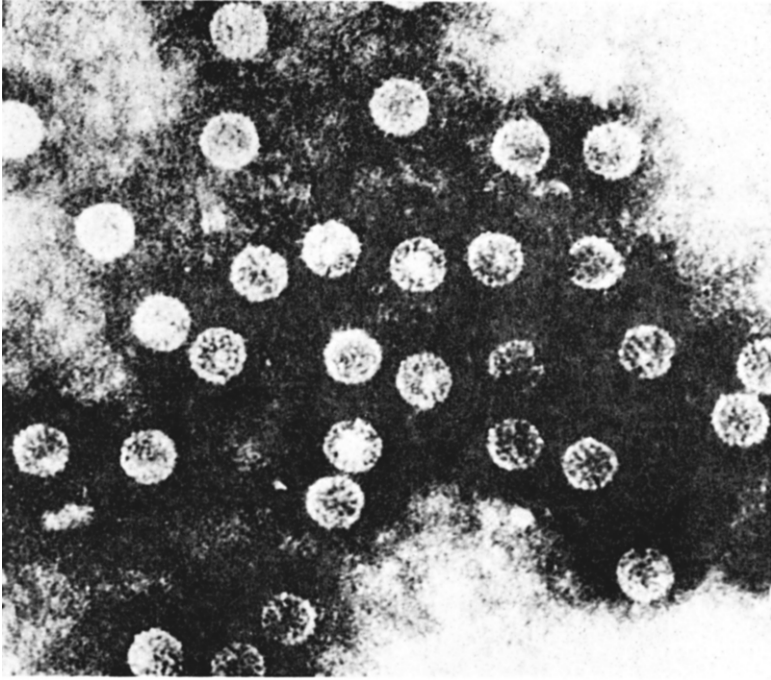
FIG. 6.6. The inner component or core of hepatitis B virus after detergent treatment with 0·5% Tween 80. × 280 000. (Electron micrograph kindly provided by Dr June D. Almeida.)

but not in the controls. These particles were either in the form of aggregates or arranged in chains. The particles displayed a hollow centre, sometimes with a definite central core. These findings have since been confirmed in many laboratories in sections of liver biopsy specimens obtained from patients with acute hepatitis B, in persistent carriers, in patients with chronic active hepatitis associated with hepatitis B virus and in patients with various liver diseases and hepatitis B surface antigen in their serum. The particles in the nucleoplasm of hepatocytes represent the core or nucleocapsid of hepatitis B virus.

After detergent treatment with 0·5% Tween 80 of pellets of antigen obtained by ultracentrifugation of whole serum, the 42-nm particles separated into an outer coat of hepatitis B surface antigen and an inner component or core, 27 nm in diameter (Fig. 6.6), which resembled morphologically a rhinovirus. Antibody in convalescent hepatitis B serum

reacted with the core to yield immune aggregates resembling those seen in homogenates of liver taken post-mortem from patients with type B hepatitis. The core antibody has an entirely different specificity from antibody to the outer (hepatitis B surface antigen) coat. An important observation was that antibody to the inner component was absent from the pre-hepatitis sera from the same convalescent patients. It was suggested that antibody to the outer coat antigen develops in hepatitis B infection but it is usually cleared from the serum with clinical improvement, while a normal immune response is produced to the core of the 42-nm particle. These observations have been confirmed in many laboratories.

The observations by electron microscopy have been extended serologically by the demonstration that all patients with hepatitis B developed complement-fixing antibodies to the core. Core antigen was fractionated from homogenized liver of a chimpanzee infected experimentally with a known infective serum containing hepatitis B surface antigen. The purified core preparation was used for the immunization of guinea pigs and a microtitre complement-fixing technique was used for measuring core antibody. Sera from guinea pigs immunized with the purified core preparation were positive at high titre (1 : 400) for core antibodies by complement-fixation but were negative for hepatitis B surface antigen by all methods including radioimmunoassay. Tests on serial serum samples from 15 patients with acute hepatitis B infection showed that antibodies to the core appeared 12–20 weeks after exposure, usually during or immediately after hepatitis B antigenaemia and well before the appearance of surface antibody. The core antibodies persisted throughout the duration of this study (up to 3 years after exposure) with a slow decline in antibody titre. Core antibodies at a titre > 1 : 128 were found in all 50 persistent carriers of hepatitis B surface antigen but none was positive for hepatitis B surface antibody when examined by passive haemagglutination. Titres of the core antibodies in three patients were not boosted by re-exposure to hepatitis B surface antigen-positive serum. The findings implied that core antibodies are produced in response to replication of the virus in the liver. Furthermore, unlike antibody to the outer antigen coat, core antibodies did not correlate with resistance to re-infection nor did they signal recovery from infection.

HEPATITIS A VIRUS

There is substantial epidemiological and experimental evidence that faeces of patients with hepatitis A are infectious approximately 2 weeks before until 2 weeks after the onset of clinical symptoms. Examination by immune electron microscopy was carried out on saline extracts of faecal specimens obtained before infection or during the acute illness from each of four adult volunteers who were inoculated either orally or parenterally with MS-1 strain of hepatitis A. Each faecal extract was diluted with saline to 2%, passed through a 450-nm Millipore membrane filter and incubated with serum of a patient convalescing from hepatitis A infection. Virus-like particles measuring 27 nm in diameter were found in faecal specimens from two out of four volunteers during the acute phase of hepatitis A. The particles were aggregated, and aggregates were composed of "full" and "empty" particles which were heavily coated with antibody. No particles were found in faecal specimens of the four subjects before they had been infected.

Faecal filtrates containing these particles were used to examine by immune electron microscopy several groups of sera for the presence of antibody to these virus-like particles. All six volunteers previously infected experimentally with hepatitis A developed serological evidence of infection as judged by aggregation and antibody coating of the 27-nm particles. A significant increase in antibody was also found in paired sera obtained from six persons from naturally occurring outbreaks of hepatitis A in Massachusetts in 1969 and American Samoa in 1972. None of the sera from the patients with hepatitis A before their exposure to the infection contained detectable antibody to the hepatitis A antigen, whereas two out of four sera obtained during the acute phase of illness did contain such antibody in low titre, suggesting that the antibody response had already begun. No serological relation was found between hepatitis A antigen and hepatitis B surface antigen and the core antigen. Similarly a serological response to the Norwalk gastroenteritis antigen could not be demonstrated with paired sera from two patients with hepatitis A, nor could a rise in antibody titre to hepatitis A antigen be demonstrated with paired sera from two volunteers infected with the gastroenteritis agent. Other studies also showed no relationship between hepatitis A antigen and the Kilham strain of latent rat virus. Two commercial preparations of pooled immunoglobulin were examined for the presence of antibody to this hepatitis A antigen. One preparation was rated by immune electron
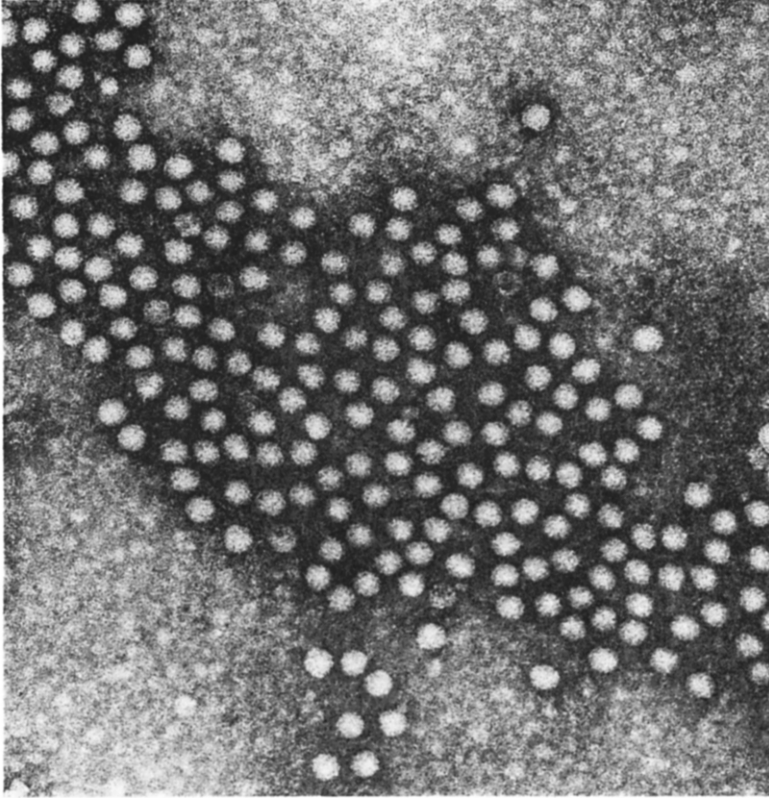
FIG. 6.7. Hepatitis A virus particles measuring 24-29 nm (average 27 nm) in diameter. Most of the particles are full. × 126 000. (From a series by A. Thornton and A. J. Zuckerman.)

microscopy as strongly positive for such antibody. It was concluded from this data that the 27-nm spherical particles visualized in faecal filtrates from patients with hepatitis A in the acute phase of the illness are the aetiological agent of this infection. These findings (Figs 6.7–6.8) have been confirmed by many laboratories, although the range of particle sizes reported is 24 to 29 nm in diameter (Thornton et al., 1977; Skidmore and Boxall, 1977).

Cook et al. (1976) described an additional morphological feature in the form of a clearly defined internal core-like structure in hepatitis A virus particles extracted from a small portion of the liver of an infected marmoset of the Saguinus mystax species. In addition, there appeared to be a
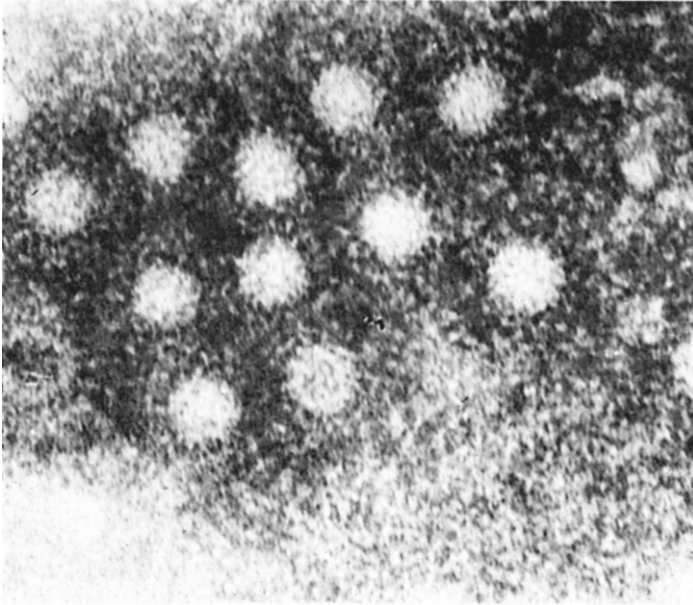
FIG. 6.8. An immune aggregate of hepatitis A virus. × 300 000. (From a series by A. Thornton and A. J. Zuckerman.)

double-layered coat around the core. If these observations are confirmed then hepatitis A virus is structurally different from the majority of enteroviruses which lack a defined core and are characterized by a single protein shell and a ribonucleoprotein core.

THE GB HEPATITIS AGENT

One of the major advances in the field of hepatitis A has been the finding of a permissive animal host for this virus (Deinhardt, 1976). However, it became clear that more than one agent was active in marmosets. The pedigree of one of these agents was established as the MS-1 strain of hepatitis A virus, while the other which was demonstrably different by neutralization and cross-challenge experiments was designated GB. The GB infectious serum was obtained on the third day of jaundice from a surgeon in Chicago who developed a mild form of acute hepatitis. This serum induced hepatitis in all four marmosets inoculated. A pool of infectious GB serum, eleventh marmoset passage, was prepared from the

total bleeding of nine white-lipped marmosets (*Saguinus* sp.) 19 to 24 days
after inoculation and during the early acute phase of hepatitis as indicated
by elevation of serum transaminase levels. Almeida *et al.* (1976) chose
serum for examination by electron microscopy from the period of maxi-
mum infectivity as established by further passage in marmosets. The
serum containing the highest infectivity titre was $10^{4.5}$ ml^{-1}. Since
immune electron microscopy has proved particularly useful in the in-
vestigation of viral hepatitis, it was decided to examine the sera in the
presence of convalescent serum from marmosets.

Examination of the infectious serum, reacted with the antibody-
containing serum, revealed the presence of scarce relatively small aggre-
gates. The particles were spherical in outline and measuring 20–22 nm in
diameter were compatible with that of the parvoviruses. However, examina-
tion of the infectious serum which had been mixed with control marmoset
serum also revealed the presence of identical immune complexes. The
implication of this finding was that the infectious serum itself contained
complexes. Subsequent examination of this serum on its own established
that this observation was correct. An immune complex was present in the
GB-positive marmoset serum. This complex reveals another distinctive
feature of this virus. Several of the particles show the rim staining associated
with empty capsids and in addition some of these empty capsids are
further fragmented to reveal crescentic forms. The morphological
characteristics of GB are in agreement with the filtration data, which
suggest that the virus is in the region of 20 nm in diameter. The second
point of interest resides in the fact that the virus is present in the serum
in the form of immune complexes. Once again this finding is in good
agreement with previously obtained data which demonstrated anti-
complementary activity of brief duration in early acute phase sera of
hepatitis A illness suggesting that immune complexes might also form in
the course of this infection. Almeida *et al.* (1976) noted that from an
electron microscope viewpoint the marmoset sera examined were near the
limits of sensitivity of direct negative staining. An infectivity titre of
$10^{4.5}$ ml^{-1} might be considered too low for visualization of virus to be
accomplished. The reason that virus was seen could well be dependent on
the fact that the majority of particles at this stage were present in the
form of immune complexes. It was also pointed out that the GB agent
does not belong morphologically to either hepatitis A or B and that it may
be another agent causing viral hepatitis.

Dienstag *et al.* (1976) used immune electron microscopy to examine

homogenates of liver obtained during the height of hepatitis induced with the GB agent. Fractions obtained by separation on a caesium chloride gradient were incubated with convalescent serum from an infected marmoset. Aggregates of virus-like particles measuring 34 to 36 nm in diameter were found at a buoyant density of $1 \cdot 4$ g ml $^{-1}$. However, when these particles were incubated with pre-infection serum, antibody was also detected. Furthermore, convalescent serum from the original surgeon (GB) did not contain detectable antibody to these particles, the implication being that these virus-like structures were not related to hepatitis.

Appleton (1977) examined by electron microscopy serum from cotton top marmosets (*S. oedipus oedipomidas*) which had been infected with the Berlin agent, a virus which is antigenically related to the GB agent. Small aggregates of spherical 22-nm virus particles were found in this serum. The particles were heavily coated with antibody and since the serum pool is highly infectious for marmosets it was presumed that free virus must exist. Similar virus-like particles were transiently excreted in the marmoset faeces, and in one animal at about the time of elevated serum transaminase levels. These findings suggested that these small virus-like particles may have a significant role in the development of Berlin and GB hepatitis in marmosets. The interpretation of these observations, however, was questioned by Dienstag (1977) who also reported the finding of morphologically similar 22-nm particles in the faeces of normal marmosets, and in pre-inoculation, acute phase and convalescent faeces from marmosets infected with hepatitis A virus.

It is difficult to determine the significance of the observations relating to the GB and Berlin agents until serological tests become available.

7

Biochemical and biophysical properties of hepatitis B surface antigen

PROPERTIES OF THE SURFACE ANTIGEN

The location of distinct surface antigen determinants on the particulate structure of hepatitis B virus has facilitated the isolation of the surface antigen from normal serum proteins in sufficient purity for immuno-chemical studies. Early studies by flotation centrifugation and the staining properties of precipitin lines indicate that the surface antigen contains both lipid and protein. The lipoprotein nature of the surface antigen allows a partial separation from other serum proteins by virtue of its characteristic buoyant density. Antigenic activity is found at a density within the range defining one of the two major subclasses of serum high-density lipoproteins (HDL_3: $1 \cdot 08 - 1 \cdot 21$ g ml^{-1}) although the two species may be readily distinguished by the much smaller diameter of the HDL_3 fraction (8–14 nm). The exact buoyant density of the surface antigen varies between sera and the chemical employed in forming the density gradient. Centrifugation of serum in buffered caesium chloride results in the separation of the surface antigen at an average density of $1 \cdot 20$ g ml^{-1}. Although the tubular forms are found in the same fraction, only a proportion of empty or partially full hepatitis B virus particles are recovered at this density. Full, or partially full, virus particles are recovered at the slightly higher density of $1 \cdot 25$ g ml^{-1} after equilibrium centrifugation in caesium chloride.
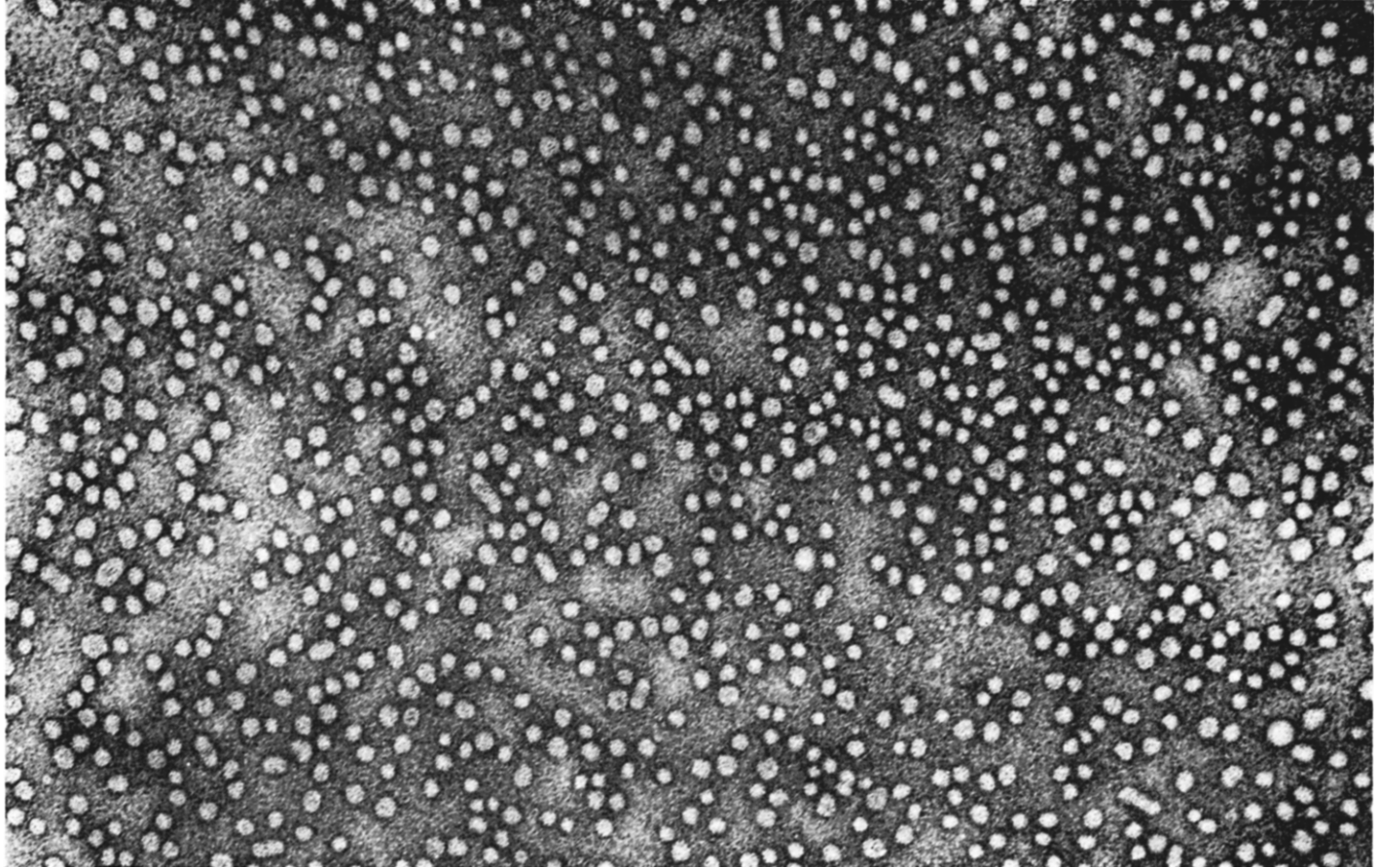
FIG. 7.1. Electron micrograph of purified hepatitis B surface antigen negatively stained with 2% phosphotungstic acid. × 100 000.

The purified small 22-nm particles, which comprise the bulk of the surface antigenic mass in most sera, have been the most common preparations used for biochemical and serological analysis. All three major morphological forms of the surface antigen may be resolved by rate zonal centrifugation, the relatively slowly sedimenting small particles having a mean sedimentation coefficient $(S_{20, w})$ in the range 33–45 S. The diffusion constant of the small particles in the analytical ultracentrifuge has given a value of 2.278×10^{-7} cm^2 s^{-1}. This value is compatible with an estimated molecular weight of 2.4×10^6, which is in good agreement with another estimate of 2.5×10^6 obtained by gel filtration.

The lipid content of the purified small particles may account for up to 30% of its total weight. Analysis of a chloroform methanol extract by thin-layer chromatography in silica gel has shown a predominance of polar lipids together with cholesterol and smaller quantities of non-polar lipids. Phosphatidyl choline, sphingomyelin and lysophosphatidyl ethanolamine were found to be the major phospholipids present. Phosphatidyl serine was absent and phosphatidyl ethanolamine could not be detected in one study.

The protein moiety of surface antigen-bearing particles has been extensively analysed in several laboratories. Examination of purified small spherical particles by ultraviolet absorption spectroscopy produces an absorption spectrum typical of protein. A substantial tryptophan content of approximately 14% may account for the somewhat high extinction coefficient at $E_{280\ nm}^{1\%} = 37.26$, although other coefficients in the range of 25–30 have also been reported. The surface antigen is also rich in hydrophobic amino acids, particularly leucine. Studies by optical rotatory dispersion and circular dichroism indicate that 70–80% of the total protein content exists as α-helix. The proline, which contributes 11.6–13.6% of the total amino acids, does not take part in α-helix formation. A similarly high α-helix content occurs in serum LDL and the filamentous bacteriophages, but other viruses in general possess 10–25% α-helix.

The treatment of purified surface antigen with organic solvents and dissociating reagents showed that the antigenic activity was stable in the presence of compounds promoting denaturation, in particular diethyl ether, 50% chloroform, urea, sodium dodecyl sulphate and various proteolytic enzymes. However, treatment with ethanol and butanol resulted in complete loss of antigenic reactivity. The surface antigen is stable for many hours at an acid pH. Treatment of serum pool by five-fold dilution with 0.02 N HCl, pH 2.3 containing 0.02% pepsin provided antigen free of normal serum proteins. Such preparations were suitable for the immunization of both guinea pigs and rabbits. It was also noted

that pre-treatment with sodium dodecyl sulphate or diethyl ether increased the susceptibility of the antigen to protective effect on antigenic determinants composed primarily of protein.

The reduction of disulphide bonds results in the complete loss of surface antigen reactivity, although considerable antigen activity may be regained by the alkylation of free sulphydryl groups with iodoacetamide. The group determinant *a* was destroyed by exposure to dithiothreitol at concentrations below 10 mM. At higher concentrations of dithiothreitol, antigenic activity which was resistant to reduction and unrelated to the *d*, *y*, *w* and *r* subdeterminants was present on the same antigen particles.

The reactivity of the surface antigen is remarkably heat-stable; no loss of antigenic activity occurs exposing purified antigen for 10 h at 60°C, although heating for 5 min at 100°C completely abolishes its affinity for antibody. Total loss of antigenic activity has been reported after 60 min incubation at 85°C. It has been demonstrated that the *a* group-specific determinant was stable at 60°C for periods of up to 21 h, whereas the *d* and *y* subtype reactivities were markedly reduced after incubation at the same temperature for only 3 h.

The stability of the surface antigen at high temperatures and its resistance to protease digestion strongly suggests the presence of carbohydrate. In addition, the precipitation of radiolabelled surface antigen by concanavalin A and a positive anthrone reaction indicate that carbohydrate may be present as well as lipid and protein. The possibility that carbohydrate may play a role in maintaining serological activity has been investigated. A 90% reduction in the serological activity of purified surface antigen particles was found after treatment with 0·1 M sodium periodate for 4 h at 37°C. A significant amount of carbohydrate relative to the protein content was found in the same preparation by the phenol–sulphuric acid method. The carbohydrate content has been estimated at 3·6–6·5% by the same method, and there is evidence that at least a proportion of the carbohydrate may be present as glycolipid. However, the possible role of carbohydrate in preserving the structural integrity of adjacent antigenic sites or carrying a novel haptenic specificity has yet to be distinguished.

Burrell *et al.* (1976) reported the results of studies using tryptic cleavage of antibody binding sites from surface antigen particles. It was found that some loss in activity occurred after treatment with periodate or with mixed glycosidases and neuraminidase, indicating a role for carbohydrate in the full expression of antibody binding activity. Reduction and

alkylation destroyed 99% of the activity. Similar results with both the released material and untreated antigen suggested that these properties were due to the structure of the antibody binding itself, rather than as a result of its involvement in the quaternary structure of the particles. In contrast, untreated antigen regained significant serological activity after reduction and dialysis to remove reducing agent, whereas the released material did not.

The polypeptide composition of the surface antigen has also been subjected to extensive analysis. At first, two major polypeptide species with average molecular weights, of 25 000 and 30 000, were described. Other components with higher molecular weight, present in variable amounts at certain stages of purification, were assumed to be contaminating serum proteins which may have a stabilizing role in preserving antigenic activity. However, further studies have shown the presence of both larger and smaller polypeptide components. Reproducible differences have been reported in the polypeptide composition of the *ad* and *ay* subtypes of the surface antigen with additional minor components present in the *ay* subtype. In another study, however, no fundamental qualitative differences were found between material of both subtypes. Some of the separated components are identifiable by the periodic acid–Schiff staining of acrylamide gels, indicating the presence of a carbohydrate moiety, which is associated with at least three polypeptides. In addition, two glycosphingolipids have been extracted which are structurally similar to the fucosylglycolipids or blood group glycolipids.

The polypeptide composition of the major surface antigen subtypes was recently compared by Shih and Gerin (1977a). Up to seven polypeptides were resolved in all the preparations examined. Two polypeptides with molecular weights of 23 000 and 29 500 respectively were consistently found as the major components. The remaining polypeptides were present in variable amounts, with their number and relative concentrations varying both between heterologous subtype preparations and within preparations of the same subtype obtained from different sources. Reduction followed by alkylation was found to have no effect on the polypeptide profiles. The most notable of the minor components was a 72 000 molecular weight polypeptide which was particularly prominent in some gels.

The major 23 000 and 29 500 molecular weight components were subsequently reported to have similar amino acid composition (Shih and Gerin, 1977b); in contrast, the 72 000 molecular weight component
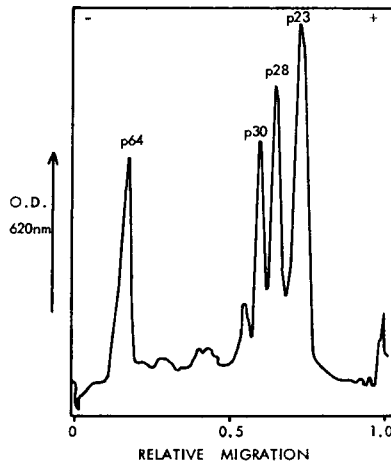
FIG. 7.2. SDS-polyacrylamide gel electrophoresis of purified HBsAg. Molecular weight estimations were derived by comparison with a parallel gel containing reference proteins of known molecular weight. The four major polypeptides identified are indicated by the prefix p and their respective mol. wt $\times$ 10^{-3} (from Skelly *et al.*, 1978).

appeared to differ significantly compared to the major polypeptides with lower molecular weights. Peterson *et al.* (1977) also found two major polypeptide species with comparable molecular weights of 22 000 and 28 000 respectively. Peterson *et al.* (1978) obtained sufficient quantities of each polypeptide by preparative gel electrophoresis to enable the identification of amino-terminal and carboxy-terminal sequences by chemical analysis, with the following sequences in each species:

(NH$_2$-terminus)
Met–Glu–Asn–Ile–Thr–Ser–Gly–Phe–Leu–Gly–Pro–Leu–Leu–Val–Ser–
Glx–Ala–Gly–Phe– · · · · · · · · · · · · – Val–Tyr–Ile
(COOH-terminus)

The common amino-terminal and carboxy-terminal sequences of the amino acids in both polypeptides strongly suggest that the surface antigen preparation consisted either of a single major polypeptide existing in both glycosylated and non-glycosylated forms or two nearly homologous polypeptides with minor differences in limited areas of their structure. Periodate–Schiff staining of analytical sodium dodecyl sulphate-polyacrylamide gels showed that the 28 000 molecular weight protein was indeed a glycoprotein. There was no staining in the region of the gel

containing the 22 000 molecular weight species. Using a different approach, Neurath *et al.* (1975) identified a 26 000 molecular weight glycoprotein by the addition of ^{14}C-sialic acid to desialyated surface antigen in the presence of sialyl transferase.

Except for the common finding of a component in the 26 000 to 28 000 molecular weight range by several laboratories, there are discrepancies in the number and size of the glycoproteins of hepatitis B surface antigen detected by selective staining of sodium dodecyl sulphate gels. This may be a result of the inconsistency of the periodate–Schiff staining method. Skelly *et al.* (1978) used the enzyme galactose oxidase for the oxidation of carbohydrate residues after removal of terminal sialic acid. A glycoprotein with a molecular weight of 28 000 was then identified as a result of the uptake of tritium label after reduction by sodium borohydride. This glycopeptide almost certainly corresponds to the glycoprotein of similar size reported by other laboratories. Further analysis showed that the radiolabel was incorporated exclusively into galactose residues, a finding which is in agreement with the data of Shiraishi *et al.* (1977) who did not detect any galactosamine in purified surface antigen. Subterminal galactose was also identified independently by Neurath *et al.* (1978) who found that desialyated surface antigen was retained on columns of immobilized peanut lectin specific for D-galactose. Skelly *et al.* (1979) using radiolabelled concanavalin A identified two glycoproteins with molecular weights of 30 000 and 32 000, in addition to the galactose-containing carbohydrate chain present in the 28 000 molecular weight component. This suggests that the additional glycopeptides contain carbohydrate chains consisting only of mannose and glucosamine.

Shiraishi *et al.* (1977) also found 4·6% N-acetylglucosamine and 0·85% sialic acid in preparations of the surface antigen. The total hexose content was estimated to be 2·5% and it was composed largely of mannose (1·21%) and galactose (0·83%). Trace amounts of the sugars fucose and glucose were also found. It was suggested that in the absence of N-acetyl-galactosamine the linkage between sugar and protein probably occurs via N-acetylglucosamine to asparagine. Owing to the apparent limited coding capacity of the viral genome, it is likely that host cell enzymes are responsible for the synthesis and addition of carbohydrate chains to the polypeptides of the surface antigen. Reference has already been made to the role of carbohydrate in maintaining serological reactivity (Burrell *et al.*, 1973) and in stimulating an effective immunological response (Neurath *et al.*, 1975).

IMMUNOCHEMISTRY OF HEPATITIS B SURFACE ANTIGEN

Several attempts have been made to define the immunological activity of individual polypeptides separated from the surface antigen by poly-acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Thus Peterson *et al.* (1977, 1978) reported that although the amino acid composition of separated 22 000 and 28 000 molecular weight poly-peptides were similar, when inoculated into guinea pigs, only the smaller, non-glycosylated component stimulated antibody to the group and subtype determinants of the complete surface antigen. In contrast, Hollinger *et al.* (1978) found that both 22 000 and 25 000 molecular weight components, prepared by a similar method, retained serological activity. Furthermore, a specific cell-mediated immune response was demonstrated in inoculated guinea pigs, and preliminary experiments in chimpanzees susceptible to hepatitis B infection showed that both preparations were capable of inducing protective antibody.

Minor components of surface antigen preparations also appear to have some specific immunoreactivity. Dreesman *et al.* (1975) isolated from purified surface antigen of subtypes *adw* and *ayw* three glycopeptides of molecular weights 19 000, 24 000 and 27 000 and two larger non-glycosylated polypeptides of molecular weights 35 000 and 40 000. Antibody and cell-mediated immune responses to the three glycopeptides of molecular weights 19 000, 24 000 and 27 000 and several larger non-glycosylated polypeptides have been studied in immunized guinea pigs. The 19 000 molecular weight polypeptide derived from *ayw* particles and the 27 000 molecular weight polypeptide from subtype *adw* and *ayw* failed to elicit an antibody response.

The polypeptides were therefore considered to contain at least the *a* group-specific determinant. However, the 24 000 molecular weight glycopeptide from both sources produced antibodies which reacted only with the homologous antigen subtype. Further studies demonstrated a cell-mediated immune response to the 24 000 and 40 000 molecular weight components (Cabral *et al.*, 1975). Peritoneal exudate cells from guinea pigs inoculated with the 40 000 molecular weight polypeptide showed a significant response when challenged with intact homologous and intact heterologous surface antigen particles. Exudate cells from animals immunized with the 24 000 molecular weight glycopeptide derived from subtype *adw* antigen responded to intact homologous antigen

and its 24 000 and 40 000 molecular weight components. A poor response to surface antigen of subtype *ayw* was observed in these animals.

Shih and Gerin (1975) were also successful in raising antibodies to polypeptide components of the surface antigen. Antisera to seven polypeptides obtained by sodium dodecyl sulphate–acrylamide gel electrophoresis of surface antigen subtype *adw* were found to react with *ad* and *ay* coated red blood cells by passive haemagglutination assay, indicating that each of the seven polypeptides possessed at least one common group-specific determinant. Competition inhibition experiments with intact *ad* particles as the competing antigen resulted in parallel slopes for the antisera. The displacement of the linear portion of the inhibition curve reflected a difference in binding affinity of these antisera for the intact surface antigen particle. Further characterization using a sensitive passive haemagglutination assay for antibody subtype analysis has shown that each polypeptide stimulated subtype-specific as well as group-specific antibodies (Gold *et al.*, 1976). Although the surface antigen preparations in both of these studies contained no demonstrable normal human serum proteins, Cabral *et al.* (1975) obtained a positive cell-mediated immune response in guinea pigs immunized with normal human serum when challenged with the 24 000 molecular weight glycopeptide isolated by Dreesman *et al.* (1975). This finding suggests that the 24 000 molecular weight glycopeptide contains at least one antigenic determinant related to certain constituents of normal human serum.

More recently Shih *et al.* (1978) isolated and labelled with ^{125}I the major structural polypeptides P1 (mol. wt 23 000), P2 (mol. wt 29 500) and P6 (mol. wt 72 000), derived from a preparation of purified 22-nm particles of the *adw* subtype of hepatitis B surface antigen. The purity and physical integrity of these labelled preparations were analysed by analytical polyacrylamide gel electrophoresis. P1 was essentially free of other polypeptides whereas P2 and P6 contained small quantities of P1, P5 and P7, respectively. The radiolabelled polypeptides P1, P2 and P6 were used as ligands in radioimmunoprecipitation tests. Antiserum titrations demonstrated that all three polypeptides were precipitated by antiserum to native surface antigen and antisera prepared against the isolated polypeptides. Therefore, each polypeptide antigen reacted not only with homologous antiserum, but also with antisera produced to the other two polypeptides demonstrating some degree of cross-reactivity between the three major polypeptides. However, the antigenicity of the isolated polypeptides was variable, the maximum precipitation of P1, P2 and P6

being 45%, 80% and 65% respectively. This variation appeared to cor-
relate with the different titres and avidities of antibodies to these poly-
peptides. The highest precipitation values were obtained from the reaction
of the individual polypeptides with their homologous antibodies. These
results could indicate structural differences between the polypeptides or
the exposure of buried antigenic sites formed during the solubilization or
renaturation procedures. However, the structural similarity of P1 and P2
would seem to favour the latter possibility. Each of the major fractions
contained both the group-specific determinant a and subtype determinant
d. Furthermore, both a surface antibody and d surface antibody activities
were isolated from antisera to each of the polypeptide fractions and shown
to react with all three polypeptide preparations. The subtype specific
antibody titres were always higher than the group specific titres for each
of the antisera to the native surface antigen. It should be noted that the
variable renaturation of antigenicity and the possible existence of buried
antigens may present problems for the use of polypeptides for active
immunization against hepatitis B virus. It was subsequently shown that
the relative immunogenicity of the various determinants present on intact
surface antigen inoculated into rabbits ranked as follows:

$$a > r > d > y >> w$$

The relative immunogenicity of surface antigen determinants were of a
different order when guinea pigs were inoculated:

$$a = d > y >> r \text{ or } w$$

Both species of laboratory animal failed to respond effectively to the
subdeterminant y. These findings thus illustrate the difficulty of assessing
accurately the immunological properties of separated surface antigen
polypeptides. Shiraishi et al. (1978) reported that both antigen determi-
nants a and d were sensitive to treatment with periodate: in contrast r and
w were unaffected by periodate suggesting that in part the laboratory
animals may respond differently to the carbohydrate moieties associated
with the surface antigen.

   In all the above studies, components of the surface antigen were
obtained after solubilization of the surface antigen with sodium dodecyl
rulphate. Although the results obtained after removal of the detergent
suggest that individual polypeptides may contain amino acid sequences in
she correct configuration for the retention of immunoreactivity, the
relatively poor affinity of the separated components for antibody does not

permit an exhaustive analysis of immunological properties. Renaturation of polypeptides into a native conformation may be incomplete or inaccurate after removal of the detergent and the antigenic determinants of such molecules may differ considerably from those in the original state. Indeed Mackay and Burrell (1976) have shown that polypeptides of hepatitis B surface antigen may undergo incomplete dissociation and reaggregation when treated with sodium dodecyl sulphate.

The antigenicity of globular proteins is dependent almost exclusively on three-dimensional conformation (Arnon and Geiger, 1977). An alternative approach has therefore been developed by Skelly et al. (1979) whereby serological activity of the surface antigen is preserved after treatment with the non-ionic detergent Triton X-100. Purified antigen was disrupted with Triton X-100 in the presence of salt to yield a product with an estimated sedimentation coefficient of 3·9. Passage of the solubilized material through a column of concanavalin A–Sepharose 4B in the presence of the detergent separated the components of the surface antigen. The first fraction, which did not bind to the lectin, contained exclusively a 64 000 molecular weight component which was identified chemically and serologically as serum albumin. This finding clearly demonstrated the presence of this host protein in purified preparations of the small particles of the surface antigen. The second fraction, recovered by elution with a-methyl-D-mannoside, contained a glycosylated 28 000 molecular weight polypeptide and a non-glycosylated 23 000 molecular weight polypeptide. Both these polypeptides were major components of the intact 22-nm surface antigen particles, and these components may possibly be joined by protein–protein linkage. The reactivity of this fraction with antisera to the surface antigen and the absence of interaction with antisera to normal serum proteins make this a suitable candidate preparation for prophylactic use (see Chapter 20). The technique of Triton X-100 solubilization followed by affinity chromatography also permits the preparation of milligramme quantities of serologically active material, which has not been possible by employing preparative sodium dodecyl sulphate gel electrophoresis.

A somewhat different approach to the identification of specific antibody binding sites was adopted by Burrell et al. (1976). Combined treatment with sodium dodecyl sulphate and trypsin resulted in the breakdown of purified surface antigen particles. Significant quantities of the group-specific determinant a were released as a glycoprotein with a molecular weight in the range of 5000 to 15 000. The serological activity of this

component resisted heat at 100°C and further proteolytic digestion indicating that non-covalent protein–protein linkage was not a major factor in maintaining the structural integrity of the *a* determinant. However, exposure to reducing agents abolished the serological activity of the separated glycopeptides, indicating the presence of disulphide bonds. Neurath *et al.* (1978) obtained slightly different results after treatment of delipidated surface antigen with sodium dodecyl sulphate and chymotrypsin. The released material from the *ad* subtype contained both *a* and *d* antigenic determinants. However, when trypsin was substituted for chymotrypsin, serologically reactive material was not released. Treatment with the enzymes subtilisin or pronase in the presence of sodium dodecyl sulphate led to the recovery of considerably less material with antigenic activity. An interesting finding was the apparent resistance of subtype *ay* to the combined effects of sodium dodecyl sulphate and chymotrypsin. The fragments released from subtype *ad* were purified further by affinity chromatography and were found to consist of several polypeptides with molecular weights of 8500 and 7800, respectively. There was no evidence of interpeptide disulphide bonds in the serologically active chymotrypsin-sodium dodecyl sulphate cleavage fragments obtained from the surface antigen. In this connection, Neurath *et al.* (1978) reported that delipidation either by extraction with chloroform : methanol mixture or tetramethyl urea was insufficient to release any protein components. This is a further indication of the importance of protein–protein interactions in maintaining both the structural and serological integrity of hepatitis B surface antigen.

TABLE 7.1

Biophysical properties of hepatitis B surface antigen small particles

Diameter	16–25 nm (by electron microscopy)
Buoyant density	
in caesium chloride	1.20 g cm^{-3}
in sucrose	1.17 g cm^{-3}
in amidotrizoate	1.19 g cm^{-3}
Diffusion constant	2.278×10^{-7} cm^2 s^{-1}
Sedimentation coefficient	30.8–40.5 S
Partial specific volume	0.739 (from amino acid composition)
Extinction coefficient ($E^{1\%}_{280\,nm}$)	37.26

Little attention has been paid so far to either the biochemical or immunochemical nature of the surface antigen which is found in the outer coat of circulating 42-nm hepatitis B virus particles. Immune electron microscopy suggests the presence of at least some common antigenic determinants between the complete virus particles and the small 20–25-nm forms of the surface antigen. Hess *et al.* (1977) demonstrated specific immunoprecipitation of the 42-nm particles with monospecific antisera to subdeterminants *d* or *y*. The possible presence of additional determinants related to the *e* antigen is discussed in Chapter 10.

Laboratory tests for hepatitis B surface antigen and antibody

Numerous laboratory methods are now available for detecting hepatitis B surface antigen and its antibody. The two-dimensional micro-Ouchterlony immunodiffusion test was the first method employed by Blumberg *et al.* (1965). Other more sensitive techniques including complement-fixation, immune adherence, electron microscopy and immune electron microscopy, various procedures of counter-immunoelectrophoresis, passive haem-agglutination and haemagglutination inhibition, radioimmunoassay, latex and charcoal particle agglutination, reverse passive haemagglutination and enzyme-linked immunosorbent assay have since been described (reviewed by Zuckerman and Howard, 1977; Maynard *et al.*, 1977).

IMMUNODIFFUSION

Immunodiffusion is based on immunoprecipitation techniques in fluid media but with the advantage that the precipitate is fixed in the supporting gel and appears as a discrete precipitin line. In general, in mixed antigen–

antibody reactions, each antigen–antibody combination will form a separate line of precipitation, distinct from those of other antigen–antibody interactions. The two-dimensional micro-Ouchterlony immuno-diffusion test, which allows direct comparisons to be made between reactions, is the simplest technique for detecting the surface antigen and the surface antibody, but, in general, it is also the least sensitive and it is slow, requiring 3 days to complete. The sensitivity of the technique can be enhanced by pre-concentration of the samples under test, by two or several fillings of the wells, or by reinforcement by placing known positive samples in wells adjacent to the specimens under test. The use of agarose instead of agar also increases sensitivity. Staining the precipitin lines will allow the detection of weak reactions. Sensitivity can also be improved by augmentation of reagent contact by rheophoresis or controlled evaporation of buffer from the surface, or by radial immunodiffusion in antibody-impregnated gel.

After primary exposure to the antigen, hepatitis B surface antibody is not usually detected by precipitin methods, but a second or repeated exposure may result in the transient development of such antibody detectable by immunodiffusion.

COUNTER-IMMUNOELECTROPHORESIS

The immunoelectrophoretic methods are essentially modifications of the electrosyneresis technique. A form of gel electrophoresis, it has been applied successfully to precipitin reactions in forensic tests for many years. The basic principle of the method is that in an electrophoretic field, antigens which migrate at alkaline pH migrate according to their res-pective net mobilities. Serum albumin, α- and β-globulins migrate towards the anode whereas the more basic gamma-globulins move towards the cathode. Under certain electrophoretic conditions in a suitable gel the two reactants will meet in optimal proportions to form a sharp precipitin line. The technique was first applied successfully for the detection of the surface antigen and antibody in 1969 and several modifications of this method have been introduced.

Counter-immunoelectrophoresis is a simple and rapid method for detection of hepatitis B surface antigen and antibody. This technique is two to ten times more sensitive than immunodiffusion for the detection of the antigen. The principal advantage of immunoelectrophoresis lies in its rapidity since the results can be obtained within 2 h. Strong positive

reactions are easily read by direct examination of the slides using oblique illumination but washing and staining are recommended. The main disadvantage of this technique is the use of a fixed antigen to antibody ratio and consequently no account is taken of the possibility of "false negative" results due to either antigen or antibody excess. It is important therefore, to determine the optimal size of wells for each antiserum by varying the sample volumes in adjacent wells of different size and shape. The use of more than one dilution of the serum under test is also important. False positive results may also be obtained if tests are carried out simultaneously for both antigen and antibody along the same electrophoretic axis.

A technique which was widely used in the past was based on the finding that dilution of hepatitis B surface antibody produced in goat in undiluted homologous (i.e. goat) normal serum resulted in enhanced sensitivity for detecting the antigen by counter-immunoelectrophoresis, using a discontinuous buffer system, compared to antibody reagent diluted in normal serum of heterologous species or in buffer solutions. It was considered that the homologous globulin stabilizes the hepatitis B globulin during migration through agarose.

Counter-immunoelectrophoresis used to be the most widely used technique for large-scale screening for hepatitis B surface antigen and antibody. The sensitivity of the technique is influenced dramatically by the quality of the reagents and technical skill. Overall, however, the method is perhaps only up to three times more sensitive than immunodiffusion and its use is no longer recommended.

COMPLEMENT-FIXATION

Attempts to utilize the complement-fixation test for the diagnosis of viral hepatitis were described over 30 years ago but the results were not reproducible. With the discovery of hepatitis B surface antigen it soon became evident that as the antigen and its specific antibody became available complement-fixation could be readily employed. Complement-fixation has been found to be nearly 25 times more sensitive than immunodiffusion for measuring the surface antigen and three times more sensitive for the assay of surface antibody. The method is also more sensitive than counter-immunoelectrophoresis for antigen detection, but technically it is more difficult to carry out. The antisera vary markedly in their suitability for detecting the antigen by complement-fixation.

Complement-fixing surface antibody is usually detected after a second or repeated exposure to the antigen and it is detectable for a period of days to weeks. The pattern of response of complement-fixing antibody roughly parallels that of antibody detected by immunodiffusion and counter-immunoelectrophoresis, but a number of precipitating antibodies that are detectable by the latter two techniques do not fix complement. The technique is no longer recommended for routine use.

INERT PARTICLE AGGLUTINATION

Detection of hepatitis B surface antigen by latex particles coated with surface antibody is a rapid and simple procedure. The test requires a minimum of equipment and time, is relatively easy to interpret, and a considerable degree of sensitivity has now been attained. Although occasional non-specific and false positive reactions are known to occur, the technique of latex particle agglutination appears useful for rapid preliminary screening. The surface antibody can be detected by its ability to inhibit latex particle agglutination.

Stevens et al. (1976) described a charcoal particle agglutination-inhibition test for hepatitis B surface antigen and antibody. The method is technically simple, the test is read in less than 90 min and its sensitivity for detecting the surface antigen approaches that of sandwich type solid-phase radioimmunoassay.

PASSIVE HAEMAGGLUTINATION AND HAEMAGGLUTINATION INHIBITION

Red cells coated with highly purified antigen, using chromic chloride as a coupling agent, are agglutinated by very small amounts of antibody, and the results may be read within 2 h. This technique of passive haemagglutination is very sensitive, being as much as 10 000 times more sensitive than the immunodiffusion technique.

However, the preparation of suitable antigen-coated red cells with chromic chloride is difficult and different lots of cells vary considerably in their sensitivity. Non-specific agglutination is frequently found with low dilutions of sera and control erythrocytes must be used. Non-specific agglutinins can be absorbed with control erythrocytes before re-testing and by heat inactivation. False negative results may be obtained with low dilutions of high-titre antibody because of the formation of prozones, and sera should, therefore, be tested at several dilutions.

Inhibition of haemagglutination by sera under test as a result of neutralization by known antibody preparations may be used for the detection of the surface antigen. The sensitivity of haemagglutination inhibition is about the same as complement-fixation.

Human blood group O, Rh-negative erythrocytes have been tanned and sensitized with purified hepatitis B surface antigen. A normal plasma sensitized cell control was prepared in parallel. The sensitized cells were used for the detection of antigen by two-stage haemagglutination inhibition. The sensitivity of the technique is reported to be similar to radioimmunoassay. A direct haemagglutination test was applied for the detection of hepatitis B surface antibody and the order of sensitivity, based on titration, was high. The procedure was modified from the conventional haemagglutination plate to a microcapillary system for testing sera for the surface antigen and antibody and the results can be read within 20 min.

Surface antigen has also been purified by adsorption to and elution from glass particles. The antigen was subsequently stabilized with dextran and adsorbed to the surface of freshly washed erythrocytes. The test can be modified for antigen detection by inhibition or by coating red cells with surface antibody.

REVERSE PASSIVE HAEMAGGLUTINATION

Reverse passive haemagglutination for detection of hepatitis B surface antigen was first described in 1969 using purified surface antibody attached to formalin-fixed erythrocytes. Since then red cells of varying species have been coated by several techniques with purified surface antibody of human or animal origin and used for the detection of surface antigen. This method has the obvious attraction of simplicity and rapidity. A number of non-specific false positive results on screening are inherent in the method due to species-specific red cell agglutinins. Confirmatory tests are therefore required and appropriate reagents are available. Reverse passive haemagglutination would thus seem to be a practical compromise between the highly sensitive but technically complex radioimmunoassay and other simple but less sensitive techniques. This test is now recommended for use as a screening test for hepatitis B surface antigen (World Health Organization, 1977).

RADIOIMMUNOASSAY

Radioimmunoassay, which combines the specificity of antigen–antibody reaction with the sensitivity of radioisotope detection, has provided extremely sensitive assays for the minute amounts of many of the peptide hormones present in plasma. All radioimmunoassay techniques depend upon the quantity of non-antibody bound antigen, non-antigen bound antibody or the extent of antigen–antibody reaction. The most frequently used procedure has been the introduction of an isotope of iodine, ^{131}I or ^{125}I, into the tyrosine residues of the peptides. Iodination with ^{125}I is more convenient due to its longer half-life and lower-energy radiation with consequent increased ease of handling.

The various radioimmunoassay procedures differ mainly according to the method of separation. The aim of separation is to resolve a reaction mixture into two portions containing free and bound reactants respectively at the end of the incubation period. Numerous techniques of separation are now available based on the ability of paper, charcoal and talc to adsorb small amounts of antigens more readily than the larger antibody molecules. Some methods utilize the size, charge and differences in solubility between antigen and antibody and there are also methods in which the antibody is fixed to Sephadex or to polystyrene by chemical or physical bonding.

The first radioimmunoassay technique for the detection of hepatitis B surface antigen and antibody by the use of the surface antigen labelled with ^{125}I was described in 1970. The sensitivity of the assay was found to be some 20 to 100 times greater than complement-fixation for detecting both antigen and antibody. A radioimmunoassay adaptation of a double antibody precipitation method to a microtitre system for detecting the surface antibody was described subsequently. This technique combines specificity with extreme sensitivity and furthermore the procedure is relatively easy and rapid. The method can also be used for assaying the antigen by the competitive blocking of the reaction of ^{125}I-labelled antigen and antibody by the addition of unlabelled antigen. The sensitivity for measuring antigen is increased in this way by 100 to 1000 times when compared with complement-fixation.

A two-step, direct, non-competitive, radioimmunoassay technique using polypropylene tubes or polystyrene beads coated with hepatitis B surface antibody raised in animals and specific hepatitis B immunoglobulin labelled with ^{125}I has been found to be more sensitive than the various

serological methods including indirect radioimmunoassays based on competition of unlabelled antigen with ^{125}I-labelled antigen. The sandwich-type radioimmunoassay technique has also been adapted for subtyping of the antigen using subtype-specific antisera. A considerable literature on the use of this commercially available method has now accumulated, and the technique has been adapted for the detection of surface antibody.

An interesting development has been the use of the protein A of *Staphylococcus aureus* for the separation of bound antigen from free antigen. Radioimmunocompetition is used for testing. Antibody is measured by direct binding of radiolabelled hepatitis B surface antigen. The principle of the technique is as follows: protein A combines specifically with the Fc-part of IgG, types 1, 2 and 4, which in normal sera comprises more than 90% of the total IgG. The reaction between protein A and the combining site on the IgG molecule is very rapid and there seems to be no difference in the combining properties to protein A between free and antigen-bound IgG. Radiolabelled antigen which reacted with its specific antibody develops, after attachment to protein A fixed to the *Staphylococcus*, into a solid phase which is readily precipitated by light centrifugation. The pellet can be counted directly without washing, once the supernatant containing the unbound radiolabelled antigen has been removed. The sensitivity for detection of the antigen is similar to that of the sandwich-type radioimmunoassay technique, and the sensitivity for measurement of antibody is at least equal to that of passive haemagglutination.

Radioimmunoassay techniques have also been used for the determination of subtypes of the surface antigen. Results with double antibody methods suggest that the number of *a* and *d* or *a* and *y* antigenic determinants per particle, or the number of antigenic sites sufficiently exposed to allow combination with antibody, may vary considerably for any given population of antigen. The results also stress a potential problem which can be encountered, particularly when sensitive techniques are used for screening of sera for the surface antigen. Antisera may contain high-titre, high-affinity antibodies to one of the type-specific *d* or *y* subdeterminants and yet fail to detect strongly positive sera of the other subtype. Furthermore, surface antigen markers may vary in their antigenic composition, thereby influencing the results of screening for antigen even with the same antisera.

A number of other radioimmunoassay procedures have also been described, including microsolid phase techniques and double antibody methods. All these methods differ primarily in the technique of separation

of the antigen–antibody complex and the placing of the label on purified antigen or purified antibody. The purity of the labelled preparation is, of course, the essential component of the specificity of the technique.

In summary, radioimmunoassay techniques include assays in which antigen–antibody complexes are separated from unbound reagents by chromatoelectrophoresis, precipitation with antibody, attachment to a solid phase or sandwich methods. Double antibody, solid-phase and sandwich systems are the most widely used techniques and provide currently the most sensitive methods for detecting hepatitis B surface antigen and antibody. Results should be confirmed as positive only if neutralization tests with surface antibody show specific inhibition (World Health Organization, 1975).

The principal disadvantages include relative slowness of the test and the high cost of capital equipment and reagents. In addition the test is tedious to perform on a large scale, the equipment is subject to breakdown and expensive maintenance and there are hazards associated with the handling of radioactive isotopes.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA OR EIA)

The use of radioisotopes for labelling reactants in immunoassays obviated the need for monospecific antisera, enhanced sensitivity and permitted the assay of haptens as well as proteins. Enzyme labelling provides a feasible alternative to radioimmunoassay and such assays provide objective results and are extremely sensitive. Although the amplification made possible by the use of enzymes to attain sensitivity will also magnify any error, advantages include the availability and relative cheapness of many enzymes and of manual and automated systems for their assay; the long shelf-life of the labelled reagent, and the lack of hazards during labelling and subsequent handling of the reagents (London, 1977).

The assay for hepatitis B surface antigen is based on a non-competitive, solid-phase procedure using disposable polystyrene microhaemagglutination plates containing sheep antibody to the surface antigen immobilized on the inner surface of each well. Bound antigen is subsequently detected by a further reaction with the gamma-globulin fraction of purified sheep hepatitis B surface antibody conjugated to horseradish peroxidase. The presence of the bound antigen is detected after the addition of a colourless enzyme substrate as a result of hydrolysis by the enzyme yielding a

coloured reaction product which is visible to the naked eye or can be read
with a spectrophotometer (Wolters *et al.*, 1976). In preliminary tests it
was found that enzyme immunoassay and radioimmunoassay for hepatitis
B surface antigen have a similar order of sensitivity and specificity
(Wolters *et al.*, 1976). These observations have now been confirmed in
many laboratories (e.g. Hansson *et al.*, 1976; Ukkonen *et al.*, 1977;
Vandervelde *et al.*, 1977). Specific confirmation of positive reactions is
based on *in situ* blocking by surface antigen bound to the solid-phase
antibody (Kacaki *et al.*, 1977).

IMMUNE ADHERENCE HAEMAGGLUTINATION

This rather complex technique has a sensitivity which is similar to reverse
passive haemagglutination. The technique consists of three steps: the
reaction of specific antibody with antigen; the addition of guinea pig
complement which results in the formation of antigen–antibody–
complement complex; and finally the adherence of this complex to
selected indicator human group O erythrocytes. This method, however,
does not detect antigen of low titre (1:2 to 1:4) nor antibody, especially
when excess serum is present in the reaction mixture. In addition, since
immune adherence is a complement-dependent reaction the same rigorous
controls and precautions employed in the complement-fixation test must
be applied. Nevertheless, this technique has been found particularly useful
for titration of hepatitis B core antibody and for hepatitis A antibody.

ELECTRON MICROSCOPY

Viruses were among the first objects to be examined in the electron
microscope over 30 years ago, but knowledge of viral fine structure was
limited to bacteriophages, until the advent in 1959 of the negative-
staining contrast technique for high resolution electron microscopy. The
technique utilizes the principle of surrounding within a rigid electron-
dense material particles such as viruses, isolated subcellular components
and other macromolecular structures. The principal advantages of negative
staining are that the technique is relatively unaffected by impurities of
small molecular size, which merge with the background, and conversely
large-sized impurities are clearly discernible from virus particles. There is
often very good preservation of material under test with minimum dis-
tortion of structure. Negative staining yields clear images and many

detailed and intricate features of particulate structures are revealed. Furthermore, only relatively small amounts of material to be tested are required. The main disadvantage of the negative-staining technique is that a high concentration of particle suspension is usually required, often 10^9 particles ml^{-1}. However, an additional degree of sensitivity may be obtained by the technique of immune electron microscopy by looking for aggregates of antigen rather than individual particles on their own. Immune electron microscopy has been especially useful for characterizing the different morphological forms of the surface and core antigens and for investigating antigen–antibody systems. Although sensitive and specific, electron microscopy is not suitable for large-scale testing.

Laboratory tests for hepatitis B core antigen and antibody

There is now substantial evidence that hepatitis B core antibody is a useful marker of infection with hepatitis B virus and persistently high titres suggest continuing viral replication, although it is recognized that this antibody remains detectable long after cessation of viral replication. Various techniques are available for demonstrating this antibody ranging from immunofluorescence to radioimmunoassay.

IMMUNOFLUORESCENCE

Fluorescent antibody techniques clearly distinguish the core antigen in the nuclei of hepatocytes. Antibody in the serum is readily demonstrated by indirect immunofluorescence using as a substrate cryostat sections of liver obtained at post-mortem from patients with a large number of core antigen-containing hepatocytes. This technique is widely used.

COMPLEMENT-FIXATION

Core antigen purified from the livers of patients obtained at autopsy or from immunosuppressed and experimentally infected chimpanzees has been used in a microtitre complement-fixation test. This method has since been automated (Coursaget et al., 1976).

Complement-fixation and counter-immunoelectrophoresis are at present the most widely used procedures for the detection of core antibody. Whilst both methods are of comparable sensitivity and specificity, relatively large quantities of core antigen are required. The conduct of these tests is therefore dependent upon a suitable source of standard antigen such as the infected liver. The advent of radioimmunoassay procedures has meant the utilization of smaller quantities of core antigen which may be readily extracted from the serum of a proportion of persistent surface antigen carriers.

IMMUNE ADHERENCE HAEMAGGLUTINATION

Immune adherence haemagglutination for the assay of core antibody is highly specific and about ten times more sensitive than the microtitre complement-fixation method (Tsuda *et al.*, 1975). The source of core antigen was complete virus particles purified on a large scale from the plasma of asymptomatic antigen carriers.

RADIOIMMUNOASSAY

A microsolid-phase radioimmunoassay technique for the detection of core antigen and core antibody was described several years ago. The wells of polyvinyl microtitre plates formed the solid phase to which was adsorbed a diluted human convalescent serum of hepatitis B. A standard core antigen preparation was prepared by the treatment of circulating hepatitis B virus particles with Nonidet P40, a non-ionic detergent found previously to be more efficient than Tween 80 for complete removal of the outer surface antigen envelope. The detection of core antibody by its ability to inhibit the binding of radiolabelled core antibody to core antigen provided a means of measuring this antibody in sera with concomitant surface antibody.

The direct radiolabelling of core antigen by activation of the endogenous DNA polymerase activity offers an alternative radioimmune procedure. A radioimmunoprecipitation technique has been described whereby antigen–antibody complexes are separated from unbound labelled antigen by precipitation with antiglobulin. A similar procedure utilizing staphylococcal protein A for the removal of immune complexes has shown that a chimpanzee serum at a dilution of 1 : 500 000 was found to precipitate 50% of added core antigen in comparison to a titre of 1 : 256–

1 : 1024 obtained by complement-fixation. For optimal sensitivity, radiolabelled core antigen is separated from both unlabelled core antigen and incompletely disrupted virus particles by equilibrium centrifugation in caesium chloride. It is often necessary to adjust the amount of radiolabelled core antigen used when different batches of antigen are employed as the specific activity of each preparation varies according to the source of plasma.

Howard and Zuckerman (1977) obtained core antigen from the sera of persistent carriers of hepatitis B virus by centrifugation and treatment with Nonidet P40 and 2-mercaptoethanol. The separated core was labelled with ^{3}H-TMP and employed in a radioimmunoassay procedure of high sensitivity for the detection of core antibody. Vyas and Roberts (1977) described a technique for the detection of core antigen and core antibody whereby the core antigen isolated from complete virus particles in plasma of a carrier and the autologous IgG labelled with ^{125}I can be used in a solid-phase radioimmunoassay. The inherent advantages of this technique also include the use of empty cores, autologous core antibody and cores as reagents, virtually eliminating non-specific reactions. The inhibition of binding of cores to the solid phase obviates the need for neutralization tests. Another solid-phase radioimmunoassay employing polystyrene beads coated with core antibody and core antigen prepared from pooled sera of persons infected with ^{125}I-labelled core antibody, was described by Neurath *et al.* (1978a).

More extensive use of radioimmune procedures for the detection of core antibody will almost certainly produce a closer estimate of the incidence of exposure to the core antigen in the blood donor population than has been hitherto possible using less sensitive techniques. In addition, radioimmunoprecipitation methods offer the advantage of allowing the estimation of core antibody levels in immunoglobulin preparations which are frequently anti-complementary when examined by complement-fixation methods.

Foreseeable developments for detection of core antibody include enzyme immunoassay procedures.

The hepatitis B *e* antigen-antibody system

It is now well established that the abundance of surface antigen produced during hepatitis B infection consists largely of non-infectious material. In particular the small 20- to 25-nm spherical particles appear to represent material surplus to virus maturation, with the larger 42-nm particles being heavily outnumbered by a ratio of a few hundred to well in excess of several thousand. Current interest in the serological diagnosis of hepatitis B is therefore directed towards the identification of those patients supporting persistent levels of virus replication and the detection of sera containing especially large numbers of complete virus particles. In both cases, hepatitis B *e* antigen has been evaluated as an immunologically distinct marker of active virus replication. The presence of hepatitis B *e* antibody (anti-*e*) indicates reduced virus activity and, in general terms, a better prognosis. At the time of writing, however, it remains unclear whether the *e* antigen is a viral gene product or whether it is primarily a product of host origin expressed only during infection with hepatitis B virus.

SEROLOGICAL DETECTION

Several serological methods are now available for the detection of *e* antigen and antibody. In order of increasing sensitivity these include immuno-

diffusion, rheophoresis, passive haemagglutination techniques, enzyme-linked immunoassay and, more recently, solid-phase radioimmunoassay. Immunodiffusion continues to be widely used for the identification of both e antigen and antibody and better results have been obtained when agarose was used at a concentration of $0.4-0.9\%$. The addition of dextran or dextran sulphate improves the clarity of the precipitation lines, an important consideration when examining the subtype specificities of this system. The use of the immunodiffusion technique for serological diagnosis has recently been reviewed (Tedder, 1978). Magnius et al. (1975) found either e antigen or antibody in 37 of 64 (58%) sera from persistently infected carriers using this simple method. Sensitivity may be marginally improved by a modification which permits a flow of buffer from the periphery of the plate towards the centre by allowing evaporation to take place from the central well. Using this method of rheophoresis, Smith et al. (1976) obtained a result for 26 of 39 (67%) sera collected from persistently infected patients. Although immunodiffusion and its modifications are relatively insensitive detection methods, it has been suggested that the majority of persistent carriers who remain unclassified by this technique are subsequently shown to possess anti-e by more recently developed methods (Tedder, 1978). The concentration of samples before testing, for example by the addition of polyacrylamide gel granules or polyethylene glycol, enhances further the usefulness of immunodiffusion. Tachibana et al. (1977) compared 100 randomly selected sera containing the surface antigen both before and after concentration. The frequency of detection of e antigen and anti-e increased from 3 to 27% and from 10 to 26% respectively after the five-fold concentration of all sera by the addition of polyacrylamide granules.

The presence of two or three parallel precipitin lines is a common feature of many reactions between sera from infected individuals and laboratory reference reagents (Fig. 10.1). The terms e_1, e_2 and e_3 antigens have been used to describe the antigenic reactivities responsible for this apparent heterogeneity as distinguished by the immunodiffusion test. However, at present there continues to be a difference between laboratories regarding the designation of each reactivity with respect to its position relative to the antigen and antibody wells. Williams and Le Bouvier (1976) reported the existence of two reactivities, which were referred to as e_1 and e_2: the e_1 antigen gave rise to a precipitation reaction close to the antibody well and the e_2 antigen produced a reaction close to the antigen well. In all sera examined, e_2 antigen always appears in
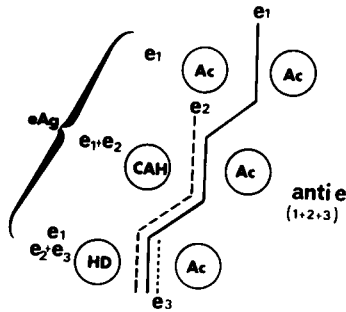
FIG. 10.1. Distribution of different e antigen specificities in various surface antigen carriers. Ac = asymptomatic carriers, CAH = chronic active hepatitis, HD = haemodialysis patient (from Trepo et $al.$, 1978).

association with e_1 although the presence of e_2 is not always detected (Tabor et $al.$, 1977; Miller et $al.$, 1978). The confusion in the terminology has followed the description of the third component e_3. Courouce-Pauty and Plancou (1978) identified e_3 as the antigenic reactivity which produces a precipitin line closest to the antibody well. This is in agreement with the smaller size and faster electrophoretic mobility of this component (Trepo et $al.$, 1978). However, a multi-centre comparison of reagents reported by Murphy et $al.$ (1978) defined the e_3 component as responsible for the reaction closest to the antigen well. These differences in terminology are summarized in Fig. 10.2. It should also be remembered that the respective concentrations of each of these components may differ between sera
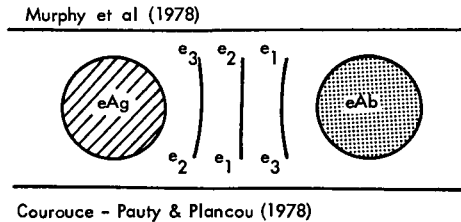
FIG. 10.2. The nomenclature of hepatitis B e antigens.

thereby resulting in an alteration of the positions of precipitation reactions with respect to the antigen and antibody wells (C. R. Howard, unpublished results).

The use of a passive haemagglutination technique for both the detection of e antigen and anti-e has been described by Takahashi et $al.$ (1977). The

reagents were prepared by firstly isolating e antigen from large volumes of plasma containing the surface antigen by affinity chromatography. This was then either used directly for the coating of sheep erythrocytes (antibody detection) or for the preparation of specific antibody in a second affinity chromatography step. This antibody was in turn used to coat red cells (antigen detection). Passive haemagglutination was found to increase the sensitivity for both antigen and antibody detection by approximately 300-fold in sera from surface antigen-positive Japanese blood donors. The e antigen was found in 21% of 80 sera tested and anti-e in a further 63%. Somewhat lower figures were obtained in a similar study of Japanese blood donors using immune adherence haemagglutination (Tachibana *et al.*, 1977). The latter method would appear to offer no significant advantage for the detection of anti-e, although prior concentration of sera up to five-fold did increase the sensitivity of this method for detection of both e antigen and anti-e.

Sandwich-type immunoassays have proved popular for the routine detection of surface antigen, surface antibody and core antibody. More recent developments for use in the e antigen system have included the use of enzyme conjugated or radioiodinated anti-e as the indicator ligand. Mushahwar *et al.* (1977) used the immunoglobulin fraction from a surface antigen-positive serum which was reactive against both e_1 and e_2 to coat polystyrene beads 6 mm in diameter. The same e antibody preparation was radiolabelled and subsequently used to detect e antigen immobilized on individual beads by immersion in the test sample. Sera suspected of
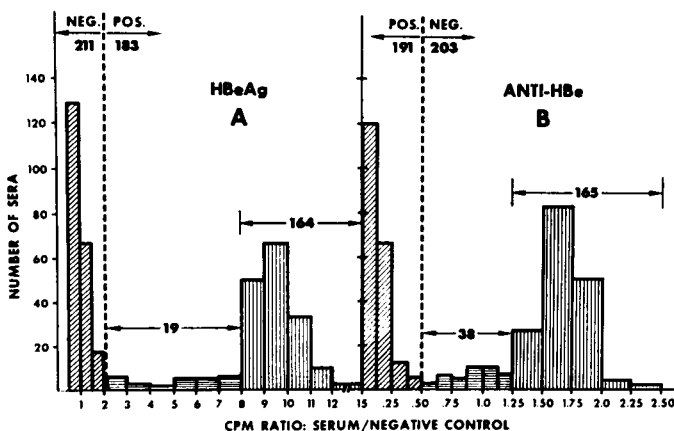
FIG. 10.3. Frequency distribution of e antigen and e antibody amongst 394 blood donors as analysed by solid-phase radioimmunoassay (from Mushahwar *et al.*, 1978).

containing *e* antigen were readily confirmed by the addition of a neutralizing serum known to contain anti-*e* before the addition of the labelled ligand. This radioimmunoassay method was found to be approximately seven times more sensitive than rheophoresis for *e* antigen and five times more sensitive for anti-*e*, the latter being detected by a competition procedure using a predetermined dilution of an *e* antigen-positive serum. The performance of the assay was examined in a total of 394 randomly selected surface antigen-positive donors (Fig. 10.3.) Of these sera, 46% were positive for *e* antigen and a further 49% were positive for *e* antibody. There was no evidence of false positive results arising from the presence of rheumatoid factor. Van der Waart *et al.* (1978) recently described the use of horseradish peroxidase-labelled *e* antibody IgG in an assay following a similar procedure using antibody-coated microtitre plates. The sensitivity of this "ELISA" method approached that of radioimmunoassay. The possibility that some false-positive reactions may occur in this system, due to the presence of rheumatoid factor, has not yet been excluded.

THE NATURE OF *e* ANTIGEN

Little information is available on the physicochemical properties of *e* antigen. Indeed it is still unclear whether *e* antigen represents material of host origin expressed only during infection with hepatitis B virus or whether this antigen is coded by the viral genome. The serologically heterogeneous nature of *e* antigen as shown by immunodiffusion analysis suggests the presence of antigenic determinants on complex molecules distinct from the morphological forms of hepatitis B surface antigen. Magnius (1975) examined the behaviour of *e* antigen separated by electrophoresis and ultracentrifugation. Sera were concentrated five-fold by the addition of polyethylene glycol 6000 and subjected to electrophoresis in agarose gels buffered from pH 4·5 to 9·5. Immunodiffusion showed a precipitate that was constantly found on the cathode side of the surface antigen precipitin, demonstrating that *e* antigen behaves as a gamma-globulin. The S value of the molecule bearing *e* antigen was estimated at 11·6 S by rate-zonal centrifugation in 10–37% sucrose gradients. Equilibrium centrifugation in caesium chloride concentrated the *e* antigen at a buoyant density of 1·291 g cm^{-3} thus confirming its protein nature.

Attempts to purify *e* antigen from serum have proved difficult owing to the apparently low quantities present in surface antigen-positive serum,

by its apparent molecular heterogeneity, and the unavailability of sensitive detection methods. Neurath and Strick (1977) partially purified the antigen by affinity chromatography using columns of immobilized e antibody. The antigen was eluted by raising the pH to 10·9, radiolabelled and subjected to further serological and chemical analysis. From the results of these studies it was concluded that the antigenic determinants resided on a structure closely resembling, or closely bound to, IgG subclass 4. Treatment of this material with 8 M urea failed to separate the antigen. The suggestion that e antigen may represent an idiotypic antibody has also been supported by the studies of Fields et al. (1978) using sera from a persistently infected chimpanzee. The e antigen was first concentrated by salt precipitation, dialysed and then further fractionated on DEAE cellulose. The antigen was recovered by elution with 0·0217 M phosphate buffer at pH 7·8 together with fibrinogen and other serum proteins. Both e_1 and e_2 specificities were present in this fraction. Gel chromatography of this preparation resulted in the elution of both specificities slightly ahead of the main IgG peak, although polypeptide species characteristic of the heavy and light chains of IgG were present. Rabbit anti-human IgG and protein A removed e antigenic activity and it was therefore concluded that the e antigen determinants were bound to, or resided on, an IgG dimer molecule. However, in both this study and that of Neurath and Strick the possibility that e antigen was a low molecular weight protein bound tenaciously to IgG was not excluded. Fields et al. (1978) found a number of unidentified minor protein components in preparations of purified e antigen.

Takahashi et al. (1977) reported that e antigen eluted immediately after the void volume when subjected to column chromatography on Sephadex G-200. A more detailed study (Takahashi et al., 1978) showed that e antigen reactivity was distributed asymmetrically with significant amounts being present in lower molecular fractions containing macroglobulin, IgG and albumin, respectively. Re-chromatography revealed that e antigen was present in the original serum as two separate molecules of different sizes. Further studies showed that the larger entity could be precipitated by the addition of ammonium sulphate to a final molarity of 1·33 and resolved further into two peaks by isoelectric focusing. The major peaks possessed an isoelectric point of 5·7 with a minor peak focusing at pH 4·8. In contrast, the smaller e antigen component was soluble in 1·33 M ammonium sulphate and possessed a single isoelectric point of 4·8. Both large and small components were also characterized by affinity chromato-

graphy using a column of immobilized anti-human IgG. When large molecular *e* antigen was applied all antigenic reactivity was bound to the column and was only recovered by elution with 3 M sodium iodide. In contrast, the smaller species of *e* antigen was not bound by anti-human IgG. These findings are summarized in Table 10.1. A line of identity was found by immunodiffusion between the two molecular forms and it was

TABLE 10.1

Summary of the properties of large and small molecular weight e antigen (Takahashi et al., 1978).

	Large	Small
Soluble in 1·33 M ammonium sulphate	No	Yes
Electrophoretic mobility	β δ	α
Size by gel filtration	Macroglobulin/IgG	IgG/albumin
Isoelectric point(s)	5·7, 4·8	4·8
Presence of IgG	Yes	No

therefore suggested that the large and small *e* antigen fractions represented immune complexes and free antigen respectively. It is possible that in other studies on the molecular nature of *e* antigen, sera containing predominantly the large form of the antigen were used. Several workers have used isoelectric focusing for separation of *e* antigen; single peaks of antigenic activity have been recovered at pH 5·0–5·7 (Neurath and Strick, 1977), and at pH 5·4 (Howard *et al.*, 1978) which may correspond to the larger, IgG-containing component of Takahashi *et al.* (1977). Neither of these components have as yet been assigned to the subspecificities of the *e* antigen system.

Reference has been made to some attempts to characterize the proteins contained in *e* antigen-positive eluates from columns of immobilized antibodies. In addition to polypeptides corresponding to the heavy and light chains of IgG, other components are frequently resolved by SDS-polyacrylamide gel electrophoresis. Howard *et al.* (1978) identified polypeptide species with approximate molecular weights of 27 000, 62 000 and 80 000. The first two components almost certainly represented the elution of small quantities of IgG from the prepared immunoadsorbent column. In addition, at least one component with a molecular weight in excess of 100 000 was visible.

It is possible that e antigen may readily form complexes with serum proteins other than IgG, notably albumin and transferrin. Figure 10.4 shows the distribution of e antigen following the separation by ion-
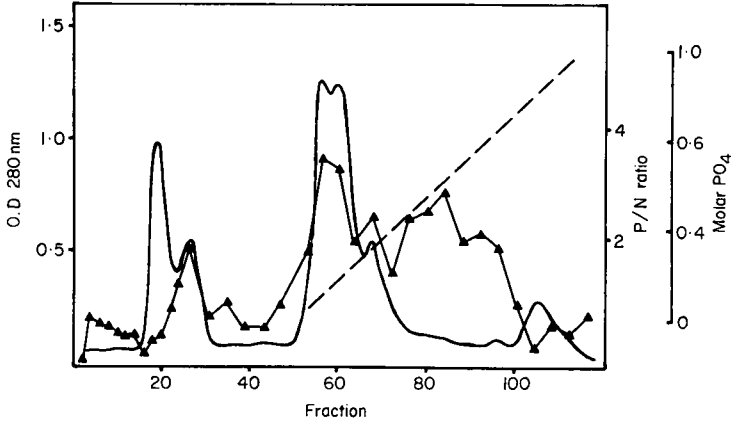
FIG. 10.4. Distribution of e antigen following the separation of hepatitis B e antigen-positive serum by ion-exchange chromatography on DEAE-cellulose. Each fraction was examined for e antigen using a solid-phase radioimmunoassay (▲–▲).

exchange chromatography of 1 ml of surface antigen-positive serum previously identified as possessing both e_1 and e_3 components. Each fraction was examined for e antigen using a solid-phase radioimmunoassay technique. In addition to the elution of e antigen together with a subclass of IgG, the antigen was also recovered along with the protein peaks containing predominantly albumin and transferrin. The bulk of hepatitis B surface antigen activity was recovered by increasing the salt concentration to 0.3 M and this was accompanied by the elution of a further fraction of e antigen. A specific association with the enzyme lactic dehydrogenase has also been reported (Vyas et al., 1977), although this finding has not yet been confirmed.

The recent development of radioimmunoassay methods for the detection of e antigen has helped overcome the difficulties associated with the purification of e antigen components, namely the close relationship with serum proteins, and the insensitivity of other techniques for the detection of the antigen in separated fractions. Neurath et al. (1978c) employed a solid-phase radioimmunoassay for the detection of e antigen after the treatment of serum with trichloroacetic acid–ethanol in order to reduce the association of the antigen with serum proteins. The antigen was

subsequently identified as having a single isoelectric point of 4·8 after extraction. Alternatively, it was found by Neurath *et al.* that *e* antigen could be purified successfully by gel filtration using 3 M sodium thiocyanate as eluate. By calibration of the gel filtration media with proteins of known molecular weight, *e* antigen was estimated to possess a mean molecular weight of 35 000. The light chain of IgG was found to be the major contaminant in these preparations. Further analysis of this material by SDS-polyacrylamide gel electrophoresis after specific immuno-precipitation with anti-*e* suggested that *e* antigen consisted of a dimer of a 17 000 molecular weight polypeptide.

Howard *et al.* (1979) confirmed the suitability of solid-phase radio-immunoassay techniques for detecting *e* antigen in separated fractions of positive serum. After concentration with 50% ammonium sulphate, two peaks of *e* antigen were resolved by gel filtration using Sepharose CL-6B. The smaller of these, with an estimated molecular weight of 130 000, was further subjected to isoelectric focusing in a shallow pH gradient. The bulk of the *e* antigen activity was recovered at an isoelectric point of 4·9. Further analysis of this material revealed the presence of two major polypeptide species with molecular weights of 66 000 and 17 000, respectively. Similarly sized structures have also been found by other workers using a combination of affinity chromatography and gel filtration (L. R. Overby, C.-M. Ling and I. Mushahawar, personal communication).

The similar size of polypeptides found associated with *e* antigen by some workers and those found in purified core antigen (see Chapter 11) stimulated preliminary experiments designed to examine the presence of *e* antigen in preparations of the 42-nm virus particles. Neurath and Strick (1979) prepared immunoprecipitates by reacting core antigen released from the 42-nm particles with core antibody. Exhaustive washing of the precipitates followed by gel filtration in the presence of 3 M sodium thiocyanate resulted in the separation of a component of similar size and serological reactivity to *e* antigen. Furthermore, antisera prepared in animals immunized with either core antigen or *e* antigen reacted with both homologous and heterologous antigens. It is therefore possible that *e* antigenic determinants may be present within the core component of hepatitis B virus distinct from those designated as core antigen. Immuno-diffusion analysis of particles treated with Nonidet P40 to release intact core components do not show any serological identity with the e_1 and e_2 components (Fig. 10.5).
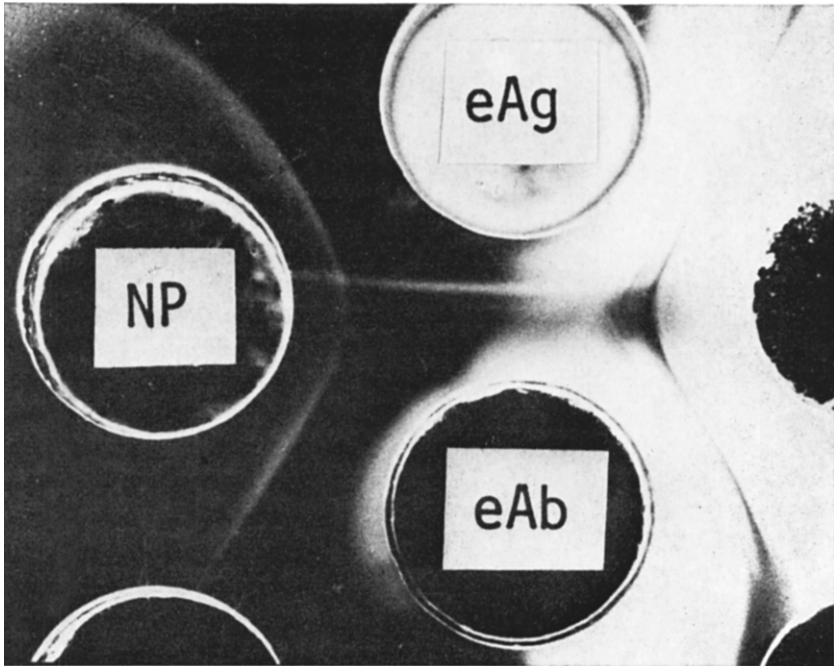
FIG. 10.5. Immunodiffusion analysis of HBV particle preparation disrupted with Nonidet P40. A line of core antigen–antibody reactivity is clearly seen between this material (NP) and both reagents in the *e* antigen system. No serological identity was seen between core antigen and the two *e* antigen precipitin lines.

POSSIBLE ASSOCIATION OF *e* ANTIGEN WITH HEPATITIS B VIRUS AND THE SURFACE ANTIGEN

Hepatitis B *e* antigen is frequently found together with significant amounts of hepatitis B virus particles in surface antigen-positive sera (Nordenfelt and Kjellen, 1975). As the complete virus particle contains a unique DNA polymerase activity, it has been suggested that *e* antigen may represent this enzyme (Melnick *et al.*, 1976). Neurath and Strick (1976) investigated this possibility by examining the behaviour of the antigen on columns containing pyran-Sepharose or immobilized DNA. Partially purified *e* antigen prepared by ion chromatography failed to bind to immobilized calf thymus DNA or to pyran-Sepharose using conditions suitable for the retention of nucleic acid polymerases. These observations indicated that *e* antigen is not a polymerase enzyme. Although it has been suggested that anti-*e* depresses DNA polymerase activity in certain sera

(Bradley *et al.*, 1976), the antibody activity is not clearly distinguished from core antibody. In addition there was no correlation between the levels of DNA polymerase activity and the presence or absence of *e* antigen in the sera tested. It is therefore unlikely that *e* antigen is related to the hepatitis B associated DNA polymerase.

The close relationship between the presence of *e* antigen and the infectivity of surface antigen-positive sera led Neurath *et al.* (1976) to examine the morphological forms of hepatitis B antigen for antigenic determinants which may be serologically distinct from surface antigen determinants. Previous studies by electron microscopy of clinical specimens showed that complexes containing either hepatitis B virus particles only or virus particles together with the tubular forms of the surface antigen were often present during infection (Field and Cossart, 1971; Moodie *et al.*, 1974). Neurath *et al.* (1976) found that partially purified virus particles, separated from free *e* antigen by ultracentrifugation, were clumped by the addition of anti-*e* to give aggregates visible by electron microscopy. The tubular forms of the surface antigen were also a frequent component of the complexes. Further evidence concerning the presence of additional unique determinants on the virus particles was obtained by affinity chromatography. Passage of hepatitis B antigen concentrates through columns of immobilized *e* antibody selectively removed hepatitis B virus particles and the tubular forms of the surface antigen. Animal antisera to these additional determinants were prepared in rabbits immunized with all three forms of hepatitis B antigen particles. Removal of surface antibody by adsorption resulted in an antibody preparation which selectively aggregated the virus particles and the tubular forms. However, other laboratories have not been able to confirm these findings. Takahashi *et al.* (1978) and Gerin *et al.* (1978) found that anti-*e* did not specifically precipitate surface antigen-containing particles from partially purified preparations of hepatitis B virus.

One explanation for these discrepancies may be that *e* antigen is cryptic in some preparations of hepatitis B virus. Lam *et al.* (1977) found that the virus particles reacted with serum containing anti-*e* only after treatment with 0·5% Tween 80, a procedure known to separate the outer surface antigen coat from the core component. However, no control data were presented in this study to exclude the possibility that the observation was not due to core antibody present in the *e* antibody-positive serum reacting with the exposed core antigen. More recently, Vnek *et al.* (1978) reported the cryptic association of *e* antigen with surface antigen-bearing particles.

The surface antigen was purified from pooled chimpanzee plasma by a combination of polyethylene glycol precipitation and isoelectric focusing. Samples isolated with 1% Tween 80 for up to 2 h led to the release of e antigen, particularly in those fractions containing virus particles and tubules. After release, e antigen was found to have an isoelectric point in the pH range of 5·6 to 6·0. This material was subsequently found to contain three polypeptides with approximate molecular weights of 25 000, 55 000 and 68 000, probably corresponding to the trace amounts of IgG and albumin, which are also found in this fraction.

More recent observations also demonstrated the association of e antigen with the virus particle. Ohori et al. (1979) found that treatment of purified virus with 0·1% Sarkosyl and 0·1% 2-mercaptoethanol released a component which was serologically identical with e antigen. Further treatment with 1% Sarkosyl in the presence of 2-mercaptoethanol resulted in the disruption of the core component and the release of more e antigen-reactive material. Centrifugation studies suggested that e antigen was preferentially associated with those particles which contained hepatitis B DNA polymerase activity. Using a different approach, Neurath and Strick (1979) chromatographed immune complexes of core antigen–core antibody on columns of Sepharose CL-4B using 3 M sodium thiocyanate as eluant. The core antigen was previously prepared from circulating virus particles and reacted with anti-core IgG prepared from an e antigen-positive serum before gel filtration. Two peaks were resolved, with the larger peak containing core antigen. The second peak reacted in radioimmunoassay only with anti-e and this peak was further characterized as a protein with molecular weight of 35 000. This observation was similar to that previously found for apparently monomeric e antigen isolated from serum and distinct from the particles associated with hepatitis B infection (Neurath et al., 1978).

Using a different approach, Werner et al. (1977) examined the relationship between e antigen and the presence of hepatitis B virus DNA in asymptomatic carriers. Of 26 surface antigen-positive sera, ten contained both e antigen and viral DNA sequences as measured by specific hybridization to a ^{32}P-DNA probe. Another ten sera in this group were positive for anti-e and only one of these produced significant hybridization. These findings are consistent with epidemiological findings which clearly demonstrate a close correlation between e antigen and infectivity of sera containing hepatitis B surface antigen.

CLINICAL SIGNIFICANCE

Hepatitis B e antigen is found only in sera containing the surface antigen. The appearance of e antigen during the acute phase of infection coincides with maximum levels of virus replication during the early stages of the illness. The e antigen frequently persists in patients who progress to chronic active or chronic persistent hepatitis. In contrast, anti-e is commonly found amongst asymptomatic carriers of hepatitis B virus with little or no histological or biochemical evidence of liver cell damage. The close association of e antigen with the pathogenicity and abnormally high levels of virus replication has promoted epidemiological investigations which link the presence of e antigen and infectivity.

Magnius et al. (1975) examined serial samples of serum from five patients infected with the MS-2 strain of hepatitis B virus. The appearance of e antigen coincided with the onset of surface antigenaemia as detected by counter-immunoelectrophoresis and preceded by several weeks the rise in serum aminotransferase levels. However, observations were not extended throughout the course of clinical illness. Howard et al. (1978) reported that both e antigen and hepatitis B DNA polymerase activity were invariably found during the first few weeks of clinical illness, but that the two markers were not necessarily present in the same serum sample. Only three out of five sera taken during the first week of illness had significant levels of DNA polymerase activity. As sensitive methods become more widely available, the numbers of sera found to contain e antigen in the absence of enzyme-associated particles will almost certainly increase. One explanation may be that e antigen appears at the same time as the onset of a transient viraemia but persists after the decline in the number of circulating virus particles.

Frosner et al. (1978) examined by solid-phase radioimmunoassay serial samples from 20 patients with hepatitis B. This technique was considered to be 500-fold more sensitive than immunodiffusion for the detection of e antigen. The antigen was found in the serum of 17 patients (85%) approximately 7 days after the appearance of dark urine and 3 days after the onset of jaundice. The antigen rapidly cleared from the serum within the first 10 days after admission to hospital and no correlation was found between the length of time of its persistence and either the presence of the surface antigen or the degree and persistence of liver damage. Anti-e was consistently present during convalescence and recovery, appearing frequently immediately after the disappearance of e antigen. Similar results

have been obtained by van der Waart *et al.* (1978) using enzyme immuno-assay.

The chimpanzee is a well-documented model of hepatitis B virus infection and it has proved particularly suitable for the study of virus-specific markers. Tabor *et al.* (1977a) examined serial samples from 35 experimentally infected chimpanzees for *e* antigen and antibody. Of these, 27 recovered from infection and the remainder became persistently infected. The *e* antigen was found by immunodiffusion in three (11%) and six (75%) respectively approximately 2–3 months after the appearance of the surface antigen but up to 1 month before biochemical evidence of liver damage. Both the e_1 and e_2 specificities were found in all cases, although tests for the presence of DNA polymerase activity were negative. During the acute infection, the appearance of *e* antigen was short-lived becoming undetectable before the disappearance of the surface antigen. Shikata *et al.* (1977) compared the infectivity of surface antigen-positive sera containing either *e* antigen or anti-*e* in susceptible chimpanzees. Serum with *e* antigen was found to be infectious when inoculated into chimpanzees at a dilution of 10^{-8}. In contrast, serum containing anti-*e* was infectious only if inoculated undiluted. In addition, the incubation period appeared to be inversely related to the titre of virus in each inoculum.

The marked increase in the frequency of progression to chronic liver disease amongst the *e* antigen-positive chimpanzees studied by Tabor *et al.* (1977a) is similar to the clinical observations on the likely outcome of hepatitis B virus infection in man (El Sheikh *et al.*, 1975; Eleftheriou *et al.*, 1975; Feinman *et al.*, 1975). Nielsen *et al.* (1976) in a detailed follow-up study of 182 patients with hepatitis B found a significant difference between patients who developed transient or prolonged anti-genaemia. Hepatitis B *e* antigen was found initially in 11 of 19 patients who subsequently developed chronic hepatitis or cirrhosis. In contrast, *e* antigen was found in only one of 41 patients who recovered. The clinical relevance of *e* antigen positivity was confirmed by significant differences in the extent of liver injury between patients positive and negative for *e* antigen during the acute phase of the illness. Similar findings were reported by Smith *et al.* (1976) who found *e* antigen in eight out of 15 surface antigen-positive patients with chronic active liver disease. However, two out of 24 asymptomatic carriers who carried the *e* antigen had no histo-logical evidence of significant liver disease. Conversely, the presence of anti-*e* in three patients with chronic liver disease shows that its presence

does not necessarily exclude the development of chronic hepatitis.

El Sheikh *et al.* (1975) examined the relationship between the appearance of *e* antigen and the presence of complete virus particles as seen by electron microscopy. Thirteen out of 17 persistently infected patients who possessed *e* antigen also had significant numbers of circulating virus particles. However, particles were also seen in the sera of another five of 27 patients who had circulating anti-*e*. These findings confirm that the presence of anti-*e* is of less importance as a prognostic marker and that its presence does not reliably exclude liver damage. Trepo *et al.* (1976a) found no evidence of *e* antigen in 43 cases of fulminant hepatitis and anti-*e* was found in only three patients. In contrast *e* antigen was found in 16 of 18 cases of polyarteritis nodosa with persistent surface antigenaemia.

The *e* antigen has been localized by immunofluorescence in the hepatocytes of patients with chronic active hepatitis. Arnold *et al.* (1977) found the antigen in the nuclei of infected hepatocytes using a double staining immunofluorescent procedure. Core antigen was frequently present in the same nuclei. This observation contrasts sharply with that of Trepo *et al.* (1976b) who found the *e* antigen largely in the cytoplasm of hepatocytes of asymptomatic carriers. At present it is not clear whether this difference is due to the different specificities of the reagents used or whether it reflects different modes of maturation of hepatitis B virus in these groups of persistently infected individuals.

Several laboratories have examined the association between *e* antigen and circulating virus particles in persistently infected blood donors and in patients treated by maintenance haemodialysis. The latter patients are known to have a particularly high risk of transmitting hepatitis B. In a study of a small number of sera, Nordenfelt and Kjellen (1975) found that almost all of the surface antigen-positive blood donors examined possessed anti-*e* with little or no evidence of circulating virus. In contrast, sera from all six patients undergoing maintenance haemodialysis contained both *e* antigen and large numbers of circulating virus particles. Imai *et al.* (1976) examined 403 sera from asymptomatic blood donors for *e* antigen and DNA polymerase. Of these sera, 14% contained both *e* antigen and DNA polymerase activity and anti-*e* only was found in 24% of the sera. The remaining samples could not be assessed for the *e* system, presumably because of the relative insensitivity of the immunodiffusion technique. However, another 15% did contain DNA polymerase activity, albeit at lower levels. Hess *et al.* (1977) found that seven out of 22 asymptomatic carriers, all with mild chronic hepatitis, also had both *e* antigen and DNA

polymerase activity. This finding suggested that such individuals repre-
sented an important source for the transmission of hepatitis B. The
remainder of the selected sera contained anti-*e* and there was evidence of
an abnormal liver histology in only one individual amongst the original
donors. There appeared to be no significant difference in the titre of core
antibody between sera with *e* antigen or anti-*e* (Takahashi *et al.*, 1976).
Although both *e* antigen and anti-*e* are found in sera with surface antigen
of subtypes *ad* or *ay*, Nash *et al.* (1978) reported a significant relationship
between subtype *ay* and the presence of *e* antigen.

Evidence on the close association between the presence of *e* antigen,
DNA polymerase and the infectivity of donor blood has been presented
in a follow-up study of 31 individuals accidentally exposed to con-
taminated syringe needles (Alter *et al.*, 1976). Hepatitis developed in 14
out of the 18 persons exposed to blood containing *e* antigen.

In contrast, hepatitis was not recorded in any of the 12 individuals
exposed to *e* antigen-negative blood. These findings suggest that blood
from carriers negative for *e* antigen carries a relatively low risk of in-
fectivity, especially when exposure involves small volumes of inocula
introduced on contaminated needles and instruments.

A relationship between the prevalence of *e* antigen among carrier
mothers and the appearance of surface antigen in their offspring has also
been demonstrated. Schweitzer *et al.* (1975) found the surface antigen
1–3 months after birth in three out of 27 infants born to surface antigen
carrier mothers. Two of the infants were born to mothers in whose sera *e*
antigen was detected. Okada *et al.* (1976) in a survey of 23 mothers in
Japan found that all babies born to *e* antigen-positive mothers developed
surface antigenaemia, whereas there was no evidence of hepatitis B in-
fection among infants born to mothers with anti-*e*. Similar findings have
been reported by Beasley *et al.* (1977) who found that 85% of babies born
to *e* antigen-positive mothers became hepatitis B carriers. Only 31% of the
babies who became persistently infected were born to *e* antigen-negative
mothers. The titre of surface antigen among the *e* antigen-positive
mothers was nearly eight times higher compared to those mothers in
whom the antigen was not detected, thereby providing further evidence
for the greater potential infectivity of blood containing *e* antigen.

Some more recent studies have shown that some sera may contain low
but detectable numbers of virus particles in the presence of anti-*e*. Such
sera have been shown to be infectious when inoculated into susceptible
chimpanzees (Berquist *et al.*, 1976; Shikata *et al.*, 1977) albeit in volumes

considerably larger than those likely to be transferred by accidental skin puncture. The assumption that anti-*e* indicates absence of virus from surface antigen-positive blood must therefore be treated with considerable caution. A comparison between the detection of *e* antigen, anti-*e* and complete virus particles by electron microscopy has shown that as many as 36% of anti-*e* carriers also possess significant numbers of hepatitis B virus particles (Trepo *et al.*, 1977). Howard *et al.* (1978) found that such sera may also contain significant levels of DNA polymerase activity.

11 | The nature of hepatitis B virus

Direct confirmation of the infectivity of the 42-nm hepatitis B virus particle has not been possible because of the lack of a suitable *in vitro* tissue culture system for the propagation of this virus, and because of the relative difficulty of purifying this particle from the other particulate forms associated with hepatitis B. However, several physicochemical properties as well as epidemiological data indicate that at least a proportion of the 42-nm particles represent the aetiological agent. These include the finding of DNA within the core of these particles, an associated endogenously activated polymerase and the morphological resemblance of the core component to other icosahedral viral nucleocapsid structures. In addition, the diameter of the presumptive virus particles corresponds closely with the early ultrafiltration experiments using sera of known infectivity. The use of nucleic acid hybridization techniques has also revealed that base sequences present in the DNA molecule are also found within infected cells. Robinson (1977) has reviewed in detail the initial experiments carried out on separated virus particles and viral components.

  The discovery of an endogenously primed DNA polymerase reaction stimulated further studies on the nature of hepatitis B virus. The properties of this enzyme are therefore described first.

HEPATITIS B VIRUS-ASSOCIATED DNA POLYMERASE

Kaplan *et al.* (1973) demonstrated a close correlation between the presence of DNA polymerase and the 42-nm virus particle. Incorporation of deoxynucleotides into an acid-insoluble product follows the removal of the outer surface antigen coat by treatment with a non-ionic detergent. The reaction proceeds in the absence of an exogenous template and it is stimulated by the presence of magnesium and ammonium ions. In contrast, the presence of manganese depresses the enzyme activity even at concentrations lower than 0·01 M. The monovalent ions sodium and potassium stimulate the reaction, and the activity of the enzyme at high salt concentrations has been used as the basis for differentiating core antigen-associated DNA polymerase from other similar enzyme reactivities (Hirschman and Garfinkel, 1977). Although it is by no means clear that hepatitis B DNA polymerase is specified by the viral genome, a comparison of the ionic requirements of the enzyme and its sensitivity to *N*-ethylmaleimide suggest that this polymerase is unique to hepatitis B infection (Table 11.1).

TABLE 11.1

Properties of core antigen-associated polymerase and other polymerases present in eukaryotic cells.

Enzyme	Na^+/K^+	Mg^{2+}/Mn^{2+}	PO_4^{3-}	*N*-ethylmaleimide
HBV	K↑ [a]	Mg↑	Inhibited	Inhibited
α	Na ↓ [b]	Mg↑	Inhibited	Inhibited
β	Na↑	Mg↑ ↑	Inhibited	No effect
γ	K↑	Mg↑	Stimulated	Inhibited

[a] = stimulation
[b] = depression

Despite the somewhat specialized nature of the assay, the detection of DNA polymerase is a useful marker of virus replication. The precise conditions required for the assay of the enzyme for diagnosis of hepatitis B have recently been reviewed by Howard (1978). Cappel *et al.* (1977, 1978) demonstrated that the presence of DNA polymerase activity is often the only marker of hepatitis B virus. Hepatitis B surface antigen was detected in 88% of 178 individuals exposed to hepatitis B and another 11% developed core antigen-associated polymerase in the absence of the surface

antigen. Hepatitis B was later confirmed in almost all of these patients after further serological examination. It has also been noted that there is frequently a close association between DNA polymerase activity and the titre of the surface antigen.

The finding of an apparently virus-specific nucleic acid polymerase with unique properties stimulated laboratory and clinical investigation of a number of antiviral compounds which might be of value in the treatment of chronic hepatitis B infection. These include leucocyte and fibroblast interferon, adenine arabinoside and others (Chapter 21). Another advantage of complementing the current serological methods is that the assay of DNA polymerase activity may discriminate between complete and defective virus particles. It has been suggested that the production of defective particles may be necessary for the establishment and maintenance of virus persistence (Gerin et al., 1975). The frequent fluctuation in the enzyme levels often observed in serial samples from persistently infected patients and the finding of apparently "empty" virus particles is consistent with this hypothesis. It is interesting that consistently high levels of DNA polymerase activity have often been noted in the serum of surface antigen-positive patients treated by maintenance haemodialysis. Possible variations occurring during the natural course of infection should therefore be considered during the monitoring of patients involved in clinical trials of antiviral agents.

Another application of the DNA polymerase reaction is the preparation of radiolabelled core antigen resulting from the close affinity of the enzyme product with the core particles. Such preparations have been used as reagents for the detection of core antibody (Greenman et al., 1975; Moritsugu et al., 1975; Howard and Zuckerman, 1977).

BIOPHYSICAL PROPERTIES OF HEPATITIS B CORE ANTIGEN

The existence of various subpopulations of hepatitis B virus particles was revealed by buoyant density analysis of sera containing large numbers of the 42-nm spherical forms. Gerin et al. (1975) found two populations of these particles by equilibrium centrifugation in caesium chloride with respective buoyant density values of $1 \cdot 19 - 1 \cdot 20$ g cm^{-3} and $1 \cdot 25$ g cm^{-3}. Somewhat higher values of $1 \cdot 24$ and $1 \cdot 27$ g cm^{-3} were reported by Hruska and Robinson (1977), but this discrepancy was almost certainly due to the different centrifugation times used by the two laboratories. In

both studies a close correlation was demonstrated between the presence of DNA, DNA polymerase activity and particles banding at the higher density. The remaining particles by these criteria may represent incomplete or defective virus particles with little or no genome content.

Removal of the outer surface antigen coat with non-ionic detergents such as Triton X-100 or Nonidet P40 results in the release of serologically reactive core antigen. Moritsugu et al. (1975) demonstrated the presence of two density populations of core particles released in this manner from circulating virus particles; the heavier particles banded at 1·35–1·36 g cm^{-3} in caesium chloride corresponding to the 110-S DNA polymerase-positive core component described by Kaplan et al. (1973). A more heterogeneous population was also recovered in the lighter-density range of 1·28–1·32 g cm^{-3} probably containing free cores without DNA polymerase in addition to cores containing DNA polymerase complexed with core antibody. It thus seems that hepatitis B virus with higher buoyant density gives rise after detergent treatment to DNA-containing core particles with DNA polymerase activity whereas the lighter band of virus particles contains predominantly low-density core components with little or no DNA content. This is consistent with the enhanced degree of penetration by negative stain observed with particles of lower density. It is probable that a third subpopulation of core antigen particles may also be present with a density intermediate between that of full and empty particles. Kaplan et al. (1978) reported a heterogeneous population of cores which band in the range 1·33–1·34 g cm^{-3}. On further analysis, these were shown to contain a lower ratio of polymerase to core activity and somewhat less than the full DNA complement found within particles banding at 1·36 g cm^{-3}.

Liver-derived hepatitis B core antigen particles were described in earlier reports as consisting of a homogeneous population of particles without DNA polymerase and with a density of 1·30 g cm^{-3}, but more recent studies suggest some heterogeneity in material prepared from infected hepatocytes. Fields et al. (1975–1976) found that a core preparation derived from a liver had more DNA polymerase activity than a similar preparation obtained from circulating hepatitis B virus particles assayed at the same concentration of particles per ml. Gel chromatography revealed that the population of cores with DNA polymerase activity derived from infected liver had a slightly higher molecular weight compared with the bulk of the core antigen. Similarly, Onda et al. (1978) demonstrated the presence of DNA polymerase-positive core particles in

infected liver which were subsequently shown to have a buoyant density of $1 \cdot 34$ g cm $^{-3}$ in caesium chloride.

Isoelectric focusing is a widely used technique for separating macro-molecules by surface charge. Using this method, Howard and Zuckerman (1977) found that the subpopulation of core antigen with DNA poly-merase had an isoelectric point of $4 \cdot 4$. Further studies by other labora-tories demonstrated the heterogeneous nature of the core antigen. Fields *et al.* (1977) found that partially purified core antigen derived from an infected liver focused at a pH of $3 \cdot 7$. Radioiodination in the presence of chloramine-T increased this value slightly to $4 \cdot 0$ corresponding closely with the value obtained with radioiodinated core antigen derived from circulating virus particles. Neurath *et al.* (1977) found that core antigen prepared from serum yielded two peaks with isoelectric points of $4 \cdot 40$ and $4 \cdot 86$, respectively. Similar results were obtained with core antigen derived from sera with or without *e* antigen, despite the finding that core antigen prepared from serum containing *e* antigen produced two peaks by equilibrium centrifugation in contrast to only one peak from serum without detectable *e* antigen. This suggests that core antigen may exist in different conformations independent of whether or not DNA is present.

CORE ANTIGEN POLYPEPTIDES

Although much attention has been devoted by many laboratories to the structure and nature of surface antigen polypeptides, relatively few attempts have been made to characterize in detail the protein composition of core antigen. In the past this has been due partly to the scarcity of suitable assays but also to the considerable difficulty of either separating sufficient quantity of the antigen from circulating virus particles or preventing the formation of immune complexes during the process of extracting core antigen from infected livers.

Hruska and Robinson (1977) used plasma which contained particularly high numbers of virus particles. The core antigen component was released from partially purified 42-nm particles by treatment with Nonidet P40 and separated further by equilibrium centrifugation in caesium chloride gradients. The material which banded at a density of $1 \cdot 38$ g cm $^{-3}$ was analysed by acrylamide gel electrophoresis after disruption with sodium dodecyl sulphate and 2-mercaptoethanol. The staining of separated com-ponents with Coomassie Blue showed the presence of a major 19 000 molecular weight component, together with two minor components of

70 000 and 80 000 molecular weights, respectively. Similar polypeptides were obtained with core antigen material recovered at the higher densities of $1 \cdot 33$ g cm^{-3} and $1 \cdot 30$ g cm^{-3}, suggesting that the association of DNA with the core antigen does not appear to require a gross change in polypeptide composition. In the same study, core antigen prepared from the liver of an infected chimpanzee also contained the same polypeptides; in addition, a larger species of molecular weight in excess of 200 000 was also observed. This species was only occasionally seen in core antigen preparations derived from circulating virus particles and may represent either a contaminant or a precursor molecule. Periodate–Schiff staining of separated components did not reveal the presence of glycoproteins at any stage.

Fields et al. (1977) similarly compared core antigen prepared either from plasma or infected liver. Analysis of polypeptides after radioiodination revealed a considerably greater number of components than reported by Hruska and Robinson (1977). Core antigen released from circulating 42-nm particles banded predominantly at an average buoyant density of $1 \cdot 33$ g cm^{-3} and was found to contain a major 79 000 molecular weight component together with 88 000 and 59 000 molecular weight polypeptides as minor components. A similar polypeptide profile was obtained with a preparation of core antigen extracted from an infected human liver. In both instances, however, additional protein was present which co-migrated with surface antigen preparations run in parallel as a control. Although cell preparations were serologically negative for surface antigen, contamination with polypeptides characteristically found in purified surface antigen remained a possibility. Budkowska et al. (1977) also analysed radioiodinated core antigen prepared from infected liver and in addition they examined the polypeptides present in immunoprecipitates of core antibody and the labelled antigen. Two major polypeptide species were resolved with molecular weights of 35 000 and 17 000. Furthermore, identical polypeptide profiles were obtained regardless of whether or not the solubilized material was subsequently alkylated with iodoacetamide prior to electrophoresis. This finding suggested that the 35 000 molecular weight component did not represent a simple dimer of the smaller 17 000 molecular weight structure found as a result of random re-oxidation of sulphydryl groups.

Neurath et al. (1978a) compared the distribution of polypeptides in core antigen separated by equilibrium centrifugation into two bands. Acrylamide gel electrophoresis of radiolabelled core antigen with a density of
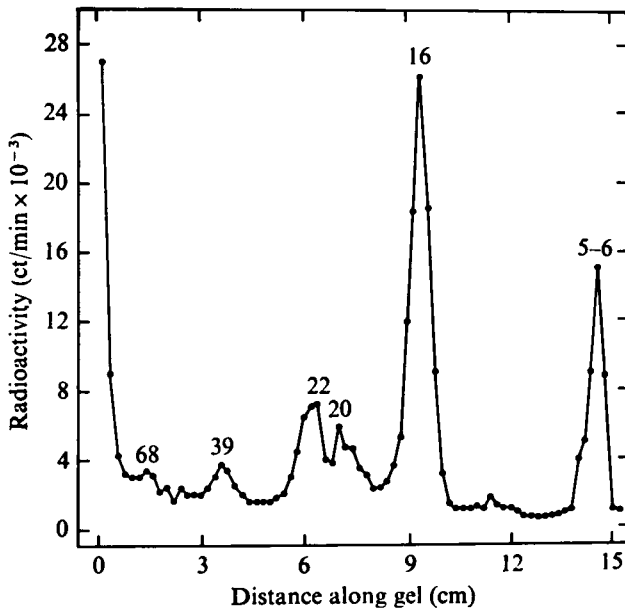
FIG. 11.1. SDS-polyacrylamide gel electrophoresis of radiolabelled core antigen derived from circulating HBV particles. Estimated mol. wts of individual peaks are shown. $\times$ 10^{-3} (from Neurath *et al.*, 1978).

1·28–1·30 g cm^{-3} in caesium chloride showed two major components with molecular weights of 16 000 and 5000 respectively (Fig. 11.1). Minor components with molecular weights of 68 000, 39 000, 22 000 and 20 000 were also resolved. Of these, the 68 000 molecular weight was much more prominent in core antigen with buoyant density 1·36 g cm^{-3}. The finding that some of the radiolabelled core antigen particles were removed on passage through a column of immobilized surface antibody was noteworthy. Subsequent analysis of the unbound fraction confirmed that the major 16 000 and 5000 molecular weight polypeptides, together with the minor 68 000 molecular weight component were specific to the core antigen.

IMMUNOCHEMISTRY OF HEPATITIS B CORE ANTIGEN

Little is known about the immunochemical properties of the polypeptides of hepatitis B core antigen found within the nuclei of infected liver cells and circulating virus particles. Budkowska (1977) studied the effect on

the core antigen of a variety of physical and chemical agents using both immunoelectrophoresis and electron microscopy. The immunoreactivity of core antigen particles prepared from the liver of a patient who died from chronic hepatitis was affected by a variety of proteolytic enzymes. Somewhat unexpectedly, both the immunoreactivity and morphology of the core particles were also affected by solvents such as 50% methanol, ethanol and butanol. In addition, the reactivity of core antigen with specific antibody was also found to be dependent on the presence of intact sulphydryl groups and possibly internal hydrophobic bonding. The morphology and immunoreactivity of the core antigen were destroyed by exposure to pH below 3·0 or by heating at 100°C.

Recent evidence concerning the nature of hepatitis B e antigen has led to the suggestion that this antigenic specificity may be closely associated with the core antigen. However, the two antigens do not give lines of identity by immunodiffusion (C. R. Howard, unpublished observation), but this does not rule out the possibility that e antigen is sequestered within the intact core antigen particle. Although it has been suggested that anti-e reacts with the core-associated DNA polymerase activity (Bradley et al., 1976), this finding has not been confirmed.

THE NUCLEIC ACID OF HEPATITIS B VIRUS

The product of the endogenously primed DNA polymerase reaction has been characterized as DNA with an approximate sedimentation coefficient of 15 S. The observation that the reaction was not stimulated by the addition of a number of synthetic or natural DNA structures indicated that the reaction template was sequestered within the core of the 42-nm virus particle (Kaplan et al., 1973). The significant inhibition of nucleotide incorporation in the presence of either actinomycin D or daunomycin also suggested that the template might be DNA. Further studies have since confirmed that at least a proportion of the separated core components contains DNA. Robinson et al. (1974) visualized circular molecules with a mean length of 0·78 μm after extraction of core particles with sodium dodecyl sulphate and mercaptoethanol (Fig. 11.2). All the circular forms seen by electron microscopy were abolished by prior incubation with DNase, although pretreatment of isolated core particles with the same nuclease did not affect the recovery of intact DNA. This latter observation provided further evidence for the sequestered nature of the DNA.
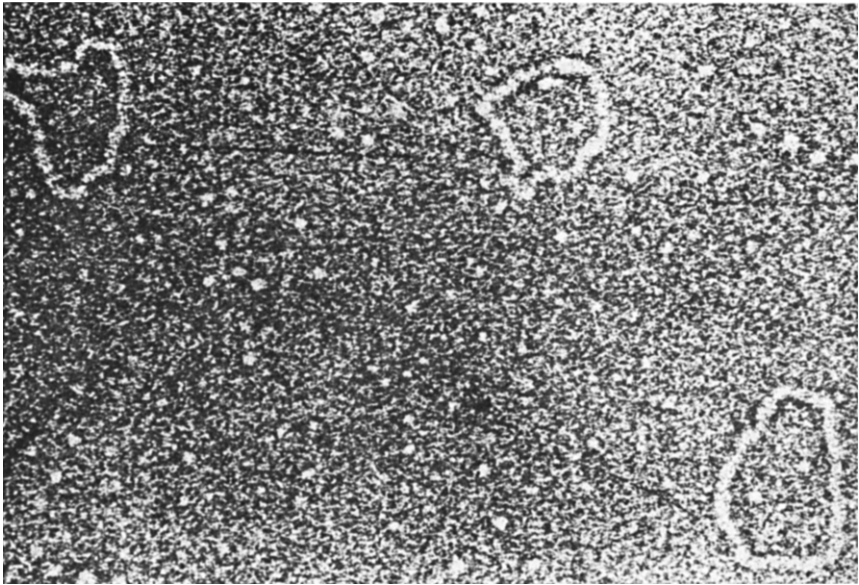
FIG. 11.2. Electron micrograph of circular double-stranded DNA molecules from HBV particles mounted by the aqueous technique (from Robinson *et al.*, 1974).

The size of the circular forms recovered by Robinson *et al.* (1974) was compatible with an estimated molecular weight of $1 \cdot 6 - 2 \cdot 0 \times 10^6$, which is considerably smaller than the genome of any other known DNA animal virus. It also appears to be smaller than the total amount of nucleic acid required to code directly for the many virus-specific antigenic determinants found in the sera of acutely and persistently infected individuals. It has been estimated that full expression of this structure would permit the synthesis of unique polypeptides with an approximate total molecular weight of 125 000 (Robinson, 1977).

The apparently unique structure of hepatitis B virus DNA and the polymerase prompted Summers *et al.* (1975) to apply restriction enzyme cleavage techniques to synthetic DNA prepared by the reaction of *Escherichia coli* DNA polymerase I with denatured core antigen DNA as the template. The high specific activity product was resolved by polyacrylamide gel electrophoresis and found to consist largely of a homogeneous 3600-nucleotide component, together with small quantities of a more rapidly migrating heterogeneous component containing a total of 3132 nucleotides. The cleavage of the major component with the restriction

enzyme endo. R *Hae III* generated 13 further fragments. The smaller heterogeneous band was found to contain between four and seven of these structures. Confirmation that the synthetic DNA product arose by the duplication in equimolar amounts of each denatured strand present in the core particle was obtained since unequal lengths of DNA were formed. Extracted DNA was incubated without prior denaturation with the polymerase of avian myeloblastosis virus. This enzyme is a nucleotidyl transferase without exonuclease activity. The incorporation of new radiolabel further confirmed the existence of substantial single-stranded regions in the native circular DNA molecule. On denaturation the native DNA molecule therefore produced two main components which behaved as linear and circular single-stranded DNA respectively. Cleavage analysis showed that the avian myeloblastosis virus polymerase reaction progressively added the remaining six fragments present on the complementary circular DNA strand by the addition of nucleotides at one or more exposed 3′-OH groups. The DNA polymerase activity associated with the intact 42-nm virus particle may therefore function as a repair enzyme by closing single-stranded regions in the otherwise double-stranded DNA structure.

Later studies concentrated on the more detailed analysis of the DNA molecule by direct examination. Hruska *et al.* (1977) estimated the frequency and size of different DNA molecular forms before and after the DNA polymerase reaction. A 27% increase in the mean length of the circles spread in an aqueous solution was observed after the enzyme reaction, which is consistent with the synthesis of complementary strands in single-stranded regions. A similar increase in the mean length of the visualized molecules was obtained by spreading unreacted DNA in formamide. The significance of circular forms with short tails in less than 10% of the observed structures was not clear, although Overby *et al.* (1975) suggested that these forms may represent structures undergoing replication by the rolling circle model for DNA replication.

Despite the experimental difficulties involved, several attempts have been made to determine whether the DNA of hepatitis B virus contains either unique virus-specific sequences or heterogeneous DNA of host origin which is incorporated at random during some stage of virus replication. Hung *et al.* (1975) demonstrated that specific hybridization could be achieved between extracted DNA and the exogenous linear DNA found free in the plasma of persistently infected carriers. No reaction was obtained with similar preparations of linear DNA made either from the

Hae III fragments of Dane particle DNA

No. base pairs

—————	A	986
- - - - -	A^1	957
- - - -	B	(800)
———————	C	415
———————	D	347
———————	E	310
———————	F	242
- - - -	G	(230)
———————	H	209
———————	I	159
———————	J	144
—————	K	111

3880 (4910)

FIG. 11.3. Diagrammatic representation of HBV DNA fragments obtained by digestion with the restriction enzyme *Hae III*. Lines represent fragments resolved by acrylamide gel electrophoresis and detected either (a) by staining with ethidium bromide (solid lines) or (b) by autoradiography after an endogenous DNA polymerase reaction (dashed lines) (redrawn from Robinson, 1977).

plasma of healthy individuals or with DNA extracted from uninfected human liver. Lutwick and Robinson (1977) extended these observations by demonstrating the specific hybridization of viral DNA to cellular DNA extracted from the liver of patients infected with hepatitis B virus. The DNA probe re-annealed with a cot $\frac{1}{2}$ value compatible with previous observations that 25–50% of the circular molecule is used as a template during the DNA polymerase reaction thereby providing further evidence on the uniformity of the template molecules. The addition of unlabelled DNA from six patients with hepatitis B infection reduced the rate of re-association by a factor of 2 or more when compared with the control reactions; thus DNA from all the infected livers appeared to contain virus-specific base sequences. It was estimated that at least 12 copies of the core-associated DNA polymerase product was present per cell DNA equivalent. This may be a conservative estimate since not every cell within the infected tissue may have been infected.

The high frequency of hepatitis B surface antigen and other markers of hepatitis B virus infection in patients with primary hepatocellular

carcinoma suggests that the viral genome may play some role in the oncogenic transformation of hepatocytes. Summers *et al.* (1978) investigated this further by examining DNA extracted from tumour, cirrhotic and metastatic tissues of four patients with primary hepatocellular carcinoma. Two of the tumours were found to contain approximately 1–2 genome equivalents per cell. Tumour tissue from a third patient with hepatitis B surface antigen was found to contain considerably fewer specific DNA sequences, and material from the fourth patient with hepatitis B surface antibody did not contain detectable hepatitis B viral DNA. It was considered possible that the positive findings may have represented the reaction of the ^{32}P-DNA probe with DNA present in the form of core or complete virus particles rather than with host cell DNA, thereby suggesting that integration of the genome of hepatitis B virus into every cell is not required for the maintenance of the neoplastic transformation of hepatocytes.

Although the isolation of the hepatitis B virus in tissue culture is yet to be achieved, the above studies suggest that the mode of replication of this virus may be unique relative to the more familiar strategies of viral genomes. A consideration of the findings by Sanger *et al.* (1977) that the genome of ϕX174 virus may code for different polypeptide species by selective initiation at different points along the virus genome may help to overcome the objection that the coding capacity of the hepatitis B viral genome does not allow for the coding of the multiple antigenic determinants expressed during infection. If this is the case, then hepatitis B virus may possess the smallest genome amongst human viruses yet recognized.

12 | Immunopathogenesis of hepatitis B

The immune response to infection with hepatitis B virus is elicited by at
least three antigenic systems, hepatitis B surface antigen, the core antigen
and hepatitis B e antigen, resulting from replication of the virus in the
liver.

THE HUMORAL RESPONSE

The surface antigen appears in the serum of most patients during the
incubation period of the acute infection, as early as 4 to 6 weeks after
infection and 2 to 8 weeks before biochemical evidence of liver damage or
the onset of jaundice. The antigen persists during the acute illness and it
is usually cleared from the blood stream during convalescence. Free core
antigen has not been detected in the circulation. Next to appear in the

circulation is the associated DNA polymerase activity immediately before or at the time of raised serum transaminase levels. The polymerase activity persists for days or weeks in acute cases and for months or years in a proportion of persistent surface antigen carriers. Antibody to the core is found in the serum 2–10 weeks after the appearance of the surface antigen and it is frequently detectable during the acute infection and for a considerable time after recovery has taken place, although with declining titres. In general, the highest titres of core antibody are found in persistent antigen carriers. Core antibody appears to be correlated with the amount and duration of replication of the virus. Antibody to the surface antigen component appears last. A primary type of surface antibody response occurs in clinical cases of hepatitis B after the disappearance of the surface antigen from the serum. In fulminant hepatitis there is some evidence to suggest that an unusually strong and rapid immune clearance of the surface antigen, associated with the early appearance of surface antibody during the peak of liver damage, may be involved in the pathogenesis of this severe form of infection (Woolf et al., 1976). The surface antibody response in most individuals who apparently resist infection on re-exposure to hepatitis B virus is of the secondary anamnestic type. The titre of core antibody, however, remains unchanged.

Anti-complementary activity, which may be associated with surface antigen–antibody complexes, may be found in the sera of some patients during the incubation period and the acute phase of illness. Such immune complexes have been seen by electron microscopy. There is now additional evidence suggesting the importance of these complexes in the pathogenesis of syndromes characterized by severe damage of blood vessels in polyarteritis nodosa, in the renal glomeruli in some forms of chronic glomerulonephritis, and in infantile papular acrodermatitis (Gianotti–Crosti syndrome). Surface antigen, surface antibody, core antibody and surface antigen/antibody immune complexes have been identified in a variable proportion of patients with virtually all the recognized chronic sequelae of acute hepatitis B. Serum anticomplementary activity and low serum complement levels may be found in patients with various forms of chronic hepatitis. Deposits of such immune complexes have also been demonstrated in the cytoplasm and plasma membrane of hepatocytes and on or in the nuclei. It is not clear, however, why circulating immune complexes are not found in a greater proportion of patients and why only a small proportion of patients with circulating complexes develop vasculitis or polyarteritis. It may be that complexes are critical pathogenic

factors only if they are of a particular size and of a certain antigen–antibody ratio. This may be an explanation of the results obtained by Reed *et al.* (1973), who studied the effects of infusion of an immunoglobulin with a high titre of hepatitis B surface antibody in six patients with active chronic hepatitis, in five of whom the surface antigen was persistently detected in the serum. The intravenous infusions were well tolerated and only two patients showed minor and transient evidence suggesting an immune complex reaction. Complexes containing hepatitis B antigen were demonstrable by electron microscopy after the infusion in four patients, but in three of these patients complexes were also present before the infusion. Complement levels and C_3 conversion products were consistent with the presence of immune complexes. The absence of any deterioration in liver function after antibody infusion suggests that, in general, immune complexes play only a minor role in the genesis of hepatocellular damage. Further studies are required to elucidate the precise role of immune complexes in the pathogenesis of liver damage (World Health Organization Report, 1977).

CELLULAR IMMUNE RESPONSES

The cytopathic effect of viruses and the immune response of the host combine to produce the clinical syndrome of a particular infection, and variations in both these factors may account for the different clinical responses. Cellular responses are known to be of particular importance in determining the clinical manifestations and course of viral infections in humans and animals. The fact that a symbiotic relationship can exist between hepatitis B virus and the host in healthy carriers suggests that the virus itself is not cytopathic, and it also implies that liver damage in patients with acute or chronic hepatitis is associated with some other indirect mechanism triggered by the virus. An analogous situation is seen, for example, in experimental infection with lymphocytic choriomeningitis virus in mice. The neurological lesions induced in this infection are considered to be mediated by a cellular immune reaction directed at viral determinants on the surface of infected cells. The lesions can be almost completely suppressed by either induction of immune tolerance to the virus, irradiation or immunosuppression with drugs. Such an explanation may account for the immunopathogenesis of liver damage in hepatitis B infection.

Studies of cell-mediated immunity in liver disease initially showed

depression of non-specific responses. The occurrence of cell-mediated immunity to hepatitis B antigens has been demonstrated by lymphocyte transformation and more particularly by leucocyte migration inhibition. When partially purified surface antigen was used as the test antigen, leucocyte migration inhibition was found in most patients during the acute phase of hepatitis B. Inhibition became less pronounced during convalescence and disappeared after recovery. Leucocyte migration inhibition has been demonstrated in a significant proportion of surface antigen-positive patients with chronic active hepatitis (Lee *et al.*, 1975). However, lymphocyte transformation and leucocyte migration inhibition were invariably negative in asymptomatic chronic carriers of the surface antigen. These observations suggest that cell-mediated immunity may be involved in terminating hepatitis B virus infection and, under certain circumstances, in promoting hepatocellular damage and may act as a link in the genesis of autoimmunity. A normal T cell function may be a prerequisite for the self-limited course of hepatitis, whereas a defective function or its absence may favour the development of chronic liver damage and the asymptomatic carrier state respectively.

Alberti *et al.* (1977) investigated the cytotoxicity of T lymphocytes to hepatitis B surface antigen-coated target cells in hepatitis B virus infection. Chicken red blood cells labelled with ^{51}Cr and coated with purified hepatitis B surface antigen using chromic chloride were employed for the detection of cytotoxic lymphocytes. The results obtained showed that cytotoxic lymphocytes were present in the peripheral blood of 65% of the patients tested 3–5 weeks after the onset of acute hepatitis B. These findings are in agreement with results obtained using other *in vitro* assay systems for cell-mediated immunity such as lymphocyte transformation and leucocyte migration inhibition. Lymphocyte cytotoxicity was detected in 33% of patients with surface antigen-positive chronic active hepatitis, but the mean cytotoxic index obtained was statistically below the value found in acute hepatitis B. Healthy carriers of the antigen failed to demonstrate cytotoxicity to target cells.

Purified T and B cell populations were prepared in eight patients with acute hepatitis B in whom unseparated lymphocytes had been shown to be cytotoxic to surface antigen-coated target cells. In all cases T lymphocyte-enriched fractions caused a significant release of isotope from target cells, while B cells were weakly cytotoxic only in one case.

Lymphocyte killing could be blocked with unlabelled surface antigen-coated chicken red cells and with purified surface antigen. The results

therefore indicate that the effector cells in this *in vitro* model are cytotoxic T lymphocytes specifically sensitized to hepatitis B surface antigen.

It has been suggested that both hepatitis B surface antigen-positive and a proportion of the antigen-negative cases of chronic active hepatitis are initiated by infection with hepatitis B virus. Testing carried out on a series of patients with chronic active hepatitis in the absence of detectable hepatitis B surface antigen provided two unexpected findings (Lee *et al.*, 1975). Examination of serum samples by electron microscopy of several of these patients with surface antibody showed the presence of immune aggregates of surface antigen in these cases. Secondly, cellular hyper-sensitivity to the surface antigen was found in more than 60% of the antigen-negative patients. This high incidence approaches that found in patients convalescing from acute hepatitis B and suggests an association between this infection and chronic active hepatitis whether or not the surface antigen is detectable (Eddleston and Williams, 1976). Indeed there are reports in the literature of progression from surface antigen-positive viral hepatitis to antigen-negative chronic active hepatitis.

AUTOIMMUNE REACTIONS

There is evidence that an autoimmune response to hepatocyte surface lipoprotein is of pathogenic importance in all varieties of chronic active hepatitis (reviewed by Eddleston and Williams, 1976) and hepatitis B virus as well as other persistent hepatitis viruses may be important aetiological agents (Lee *et al.*, 1977). A genetic factor may also be involved, as indicated by an association with histocompatibility antigens HL-A1 and HL-A8 and the increased frequency of serological abnormalities in first-degree relatives. Non-organ specific tissue antibodies commonly detected in the sera of patients with active chronic hepatitis may also be present transiently in acute viral hepatitis, and their production is likely to have been stimulated by antigens released from damaged liver cells. Liver specific antibodies directed at cell-surface determinants have not been detected in serum, but immunoglobulin has been identified on the cell membrane of hepatocytes from patients with the active disease. A liver specific lipoprotein has been identified and it seems to be a normal constituent of the hepatocyte cell membrane. Cell-mediated immunity to this lipoprotein was found in 92% of untreated patients with active chronic hepatitis. Sensitization to a surface antigen might be expected to be damaging to hepatocytes, and killing of isolated rabbit hepatocytes

when incubated with lymphocytes from 20 out of 22 cases has been demonstrated. Blocking experiments strongly suggest that the cytotoxicity is due to an immunological reaction directed at this cell surface antigen.

Interactions between T and B lymphocytes are considered to be of fundamental importance in the development of autoimmunity. B lymphocytes responsive to many of the self-antigens are present in normal persons but are only activated to produce antibodies when T cells provide a "helper effect". Autoreactive T cells are normally rendered unresponsive by small amounts of tissue antigens in the circulation, but T and B cell co-operation, with production of autoantibody, can occur if a new antigenic determinant is introduced on or close to a self-constituent, to which other T cells can respond. Evidence has recently been obtained that a damaging autoimmune B cell response may be important in the development of active chronic hepatitis. Using the isolated rabbit hepatocyte preparation, it was shown that a T cell-depleted fraction from the peripheral blood of patients with active chronic hepatitis is as cytotoxic as the original T and B population.

The working hypothesis suggested by Eddleston and Williams (1974) is that both hepatitis B surface antigen-positive and many of the antigen-negative cases of chronic active hepatitis are initiated by exposure to hepatitis B virus. The virus penetrates some of the hepatocytes, and, towards the end of the incubation period, virus-associated antigens appear on the surface of infected cells. The frequent finding of hepatitis B surface antigen in the membrane of hepatocytes as well as in the cytoplasm of persistently infected liver cells, and its increased presence in immuno-suppressed patients (Ray et al., 1976; Busachi et al., 1978) supports this view. T cells recognizing these new determinants destroy the infected hepatocytes. Virus is released and in turn infects other hepatocytes. This is normally prevented by the production of hepatitis B surface antibody. But, those patients who progress to antigen-positive active chronic hepatitis either fail to produce sufficient antibody or low-affinity antibody. The infection then persists.

Another effect of T cell stimulation is the activation of B cells responsive to existing hepatocyte cell surface antigens, including the liver specific lipoprotein. Antibodies to this antigen are produced, and on entering the liver, bind to the surface of the periportal hepatocytes. While fixation of complement is one possible mechanism leading to necrosis, it is likely that K cells would also be activated. These cells have receptors

on their surface for the Fc portion of antibody molecules, and are cytotoxic when bound to antibody-coated cells.

The synthesis and release of damaging autoantibody would normally be subject to control by suppressor T cells. But, in antigen-positive active chronic hepatitis this is not achieved, despite normal suppressor T cell function, because of the continuous activation of helper T cells. In hepatitis B surface antigen-negative cases suppressor T cell function is defective, and although the helper T cell effect is only transient, the autoimmune response persists.

One of the predictions of this hypothesis is that the autoimmune liver damage is antibody-mediated, either directly or by the activation of K cells, and that there are no effector T cells directed at unaltered liver specific lipoprotein. By removing either E or EAC rosetting cells from mixed peripheral blood lymphocytes of patients with chronic active hepatitis, enriched T and B cells are obtained which can be tested for cytotoxic activity on isolated hepatocytes. The results indicate that the effector cells in this system are able to form EAC rosettes, and this together with the observations of the blocking effect of non-specific immune complexes and aggregated immunoglobulin suggest that they are K cells.

THE ROLE OF CORE ANTIGEN IMMUNE COMPLEXES

The role of immune complexes of hepatitis B core antigen in the patho-genesis of acute and chronic liver damage has been investigated by several groups. Rizzetto *et al.* (1976) found core antigen by direct immuno-fluorescence in a large number of patients with chronic liver disease and circulating hepatitis B surface antigen, and in three patients from whom biopsies were obtained during the incubation period of acute hepatitis B. In all the biopsies from patients with chronic liver disease and core antigen, a strong nuclear and, less frequently, weaker cytoplasmic complement binding activity was observed, sometimes associated with nuclear deposits of IgG. IgG, when present, was always located only in core-positive nuclei, as shown by double staining with anti-core conjugated with fluorescein isothiocyanate and anti-IgG conjugated with rodamine iso-thiocyanate. Using the same technique it was shown that surface antigen, when present in core-positive biopsies, was often expressed in complement-fixing cells.

The inverse relationship often observed between *in vitro* complement-

fixation and direct core immunofluorescence may be explained by a variable masking by immunoglobulins of core determinants that are no longer available to the specific antiserum. There is, therefore, indirect evidence suggesting that core complement-fixing immune complexes are formed in the liver of patients with chronic surface antigen-positive hepatitis, and are somehow linked to the activity and progression of the disease. Whether IgG molecules enter an intact or damaged hepatocyte or are taken up as an immune complex with core antigen, and how they get across the plasma membrane to the nucleus, is not known.

Neither complement binding nor IgG deposits are observed in acute self-limited hepatitis B and in serologically positive patients with normal liver or minimal histological lesions, with and without hepatitis B surface antigen cytoplasmic fluorescence in their liver biopsy.

Gerber et al. (1976) examined liver specimens from patients with circulating surface antigen and core antigen in the nuclei of hepatocytes by immunofluorescence for immune complexes. A striking difference of staining patterns was observed between 11 cases with chronic active hepatitis with or without cirrhosis and ten patients with acute viral hepatitis or massive hepatic necrosis.

In chronic active hepatitis the nuclei of many of the hepatocytes showed distinct granular staining after incubation with core antibody. In nine out of 11 cases, 50% or more of the nuclei fluoresced. In parallel sections, many of these nuclei also contained IgG in a granular distribution. The nuclei did not stain directly with anti-β_{IC}/β_{IA}, indicating the in vitro binding of complement. The cytoplasm of a few hepatocytes of nine patients with chronic active hepatitis contained surface antigen, but no immunoglobulins or complement. In contrast, core antigen was found in only a few scattered nuclei in the ten specimens from patients with acute hepatitis or massive hepatic necrosis. The staining was weaker and not as granular as in the group with chronic active hepatitis. Immunoglobulins and in vitro bound complement were not detected in the nuclei. Scattered interstitial cells, probably Kupffer cells, were stained by core antibody in three specimens. Surface antigen was detected in the cytoplasm of hepatocytes in three cases, two of which were cases of massive hepatic necrosis.

Core antibody was detected by indirect immunofluorescence in the serum of all the patients with chronic active hepatitis, and in all but one of the patients with acute hepatitis.

These findings also suggest that intranuclear IgG has core antibody

specificity in chronic active hepatitis and forms immune complexes with hepatitis B core antigen. The binding of IgG to intranuclear core antigen may be of pathogenic importance in chronic active hepatitis.

As far as the core antigen is concerned, cell-mediated immunity to this antigen as measured by leucocyte migration inhibition has been found twice as frequently as inhibition to the surface antigen in patients with acute hepatitis B, in convalescent patients and in patients with chronic hepatitis in whom surface antigen and/or core antibody have been found (World Health Organization Report, 1975). Studies of *in vitro* cell-mediated immunity are more complex and difficult to carry out than those involving the humoral immune response. Reservations remain on the interpretation of the results and more studies of cell-mediated responses in viral hepatitis are needed to clarify their role in the pathogenesis of the disease.

EXTRAHEPATIC LESIONS IN HEPATITIS B

An immunological mechanism is also strongly implicated in the extra-hepatic manifestations of hepatitis B and the formation of immune complexes between specific viral antigen and host antibody bears a close resemblance to classical serum sickness. Immune aggregates are formed as a result of the specific interaction of antigens and their homologous anti-bodies, and their ability to produce tissue injury is largely dependent on the ratio of antigen to antibody and on complement. Immune complexes formed in the presence of excess antibody are usually insoluble and they are rapidly eliminated. Complexes formed in the presence of excess antigen are usually soluble and as such they tend to remain in the circulation, from which they may be deposited in certain sites such as the renal glomeruli, the skin, the walls of small blood vessels and the synovial tissues. After deposition these complexes show a high affinity for complement leading to inflammatory and other changes associated with the local aggregation of polymorphonuclear cells and the release from their lysozymes of lytic enzymes.

ARTHRITIS

Investigation of patients with acute hepatitis who had symptoms of arthritis and/or an urticarial rash has shown a good correlation between high titres of hepatitis B surface antigen and low complement levels,

which is suggestive of the presence of complement-fixing immune complexes. The total serum haemolytic complement (CH_{50}) and C4 tended to be severely depressed in the patients investigated during the acute phase, C3 levels were moderately reduced and C1q levels varied widely. The C1 inhibitor and C9 concentrations were normal. During remission of symptoms or during convalescence from viral hepatitis CH_{50} and C3 serum levels were found to be normal.

There are two possible mechanisms for acute arthritis resulting from viral infection. Firstly, the lesions could be induced by direct action of the virus on the synovial tissue. Secondly, and probably more importantly, the lesions may result from the formation of immune complexes and not necessarily from the direct cytopathic activity of the virus itself. This in turn suggests that any viral antigen could induce a rheumatoid-type of arthritis once the virus had gained access to the synovial tissue. It appears that both mechanisms can play a part in the arthritis of hepatitis. Schumacher and Gall (1974) examined for evidence of hepatitis B the synovial effusion and synovial tissue obtained by needle biopsy from a patient with acute hepatitis and a patient with chronic active hepatitis. Both patients had circulating hepatitis B surface antigen. The synovial effusion in the patient with acute hepatitis was inflammatory in nature, with neutrophils predominating. Synovial fluid was not obtained from the patient with chronic active hepatitis. There was little evidence of inflammatory cell infiltration in the many pieces of synovial membrane examined from both patients. The unusual prominence of lining cell processes and evidence of vascular damage suggested these two sites as the likely targets. Examination by the immunofluorescent antibody technique of the synovial membrane from the patient with chronic active hepatitis suggested the presence of hepatitis B surface antigen but without any localization to specific structures. Electron microscopy of thin sections revealed virus-like particles measuring 20–25 nm in the nuclei and cytoplasm of the endothelium of blood vessels, synovial lining cells and other deep synovial cells. Particles measuring 40 nm in diameter were also identified but these were also mixed with particles varying in size up to 60 nm.

It was suggested that direct virus infection of the synovial cells could have caused the arthritis. Other viruses, for example rubella, have been shown to invade the synovium and to persist in joints for up to 4 months. The necrosis of many cells containing virus-like particles in the two patients examined suggested that direct virus invasion may have damaged

at least some cells. The rarity of lymphocytic infiltration of the synovium suggests that cell-mediated immune mechanisms are not involved in this type of arthritis.

POLYARTERITIS NODOSA

Hepatitis B surface antigen has been found in the serum of a significant proportion of patients with the typical features of polyarteritis nodosa in whom the diagnosis was confirmed by muscle and liver biopsies. Some of the patients also have evidence of mild liver damage. Deposits of hepatitis B surface antigen have been demonstrated by immunofluorescence in muscle tissue and in blood vessel walls adjacent to the elastica interna. Similar arterial fluorescence was found on staining with anti-IgM and anti-β_{1C}, and circulating surface antigen–antibody immune complexes have been found in serum. These observations suggest that hepatitis B immune complexes play an aetiological role in the pathogenesis of diffuse systemic vasculitis in some patients. It is noteworthy that the concept of hepatitis B virus playing a causative role in polyarteritis nodosa is consistent with the existence of animal models in which similar forms of vasculitis are present in chronic viral diseases, for example, Aleutian disease of mink and equine polyarteritis.

Trepo et al. (1974) examined sera from 55 patients with histologically confirmed polyarteritis nodosa and found no definite correlation between clinical and laboratory indicators of polyarteritis and detection of circulating immune complexes. Seroconversion or the presence of hepatitis B surface antibody alone were associated with a better prognosis. The observation that the titre of the surface antigen may fall during exacerbation of illness and that clinical improvement may coincide with disappearance of antigen and appearance of antibody is compatible with the hypothesis of an immune complex mechanism for the pathogenesis of polyarteritis nodosa. Such a mechanism is also suggested by analogy with much of the symptomatology, for example, of arthralgia, myalgia, urticaria and glomerulonephritis and the characteristic features of human or experimental serum sickness. Polymorphonuclear leucocytosis, eosinophilia and the histological features of the vasculitis are also suggestive of an Arthus-like phenomenon with fibrinoid necrosis. Such an interpretation is in agreement with the demonstration by immunochemical means of immuno-globulins and complement at the site of tissue injury. However, it is not clear why circulating hepatitis B antigen–antibody complexes are not

found in a greater proportion of patients and why only a small proportion of patients with circulating complexes develop vasculitis or polyarteritis. The factors which determine the localization and inflammatory effects of immune complexes responsible for the development of vasculitis or glomerulonephritis are not fully known. It seems that complexes are pathogenic only if they are of a certain size and definite antigen–antibody ratio. The nature of the antibody may also be critical. Further study is therefore required to determine the role of non-circulating locally produced immune complexes involving hepatitis B antigens and to investigate the possible role of other immune complexes related to hepatitis B virus, but distinct from the characteristic particles of hepatitis B surface antigen.

GLOMERULONEPHRITIS

The predominant immunological mechanism causing human glomerulo-nephritis involves the deposition of circulating antigen–antibody complexes within the capillary walls of the renal glomeruli, which lead in turn to an inflammatory response and pathological alterations in the glomeruli. However, a relevant antigen has been implicated in only a few patients with immune complex glomerulonephritis.

Brzosko et al. (1974) identified immunoglobulins and complement by immunofluorescence in 32 of 52 unselected kidney-biopsy specimens from children with clinical nephrosis and/or glomerulonephritis admitted to hospital in Poland. Hepatitis B surface antigen was detected by direct immunofluorescence in the renal glomeruli in 18 (56%) of 32 unselected biopsies. The identification of the surface antigen in the glomerular deposits of immunoglobulins and complement was confirmed by blocking and adsorption procedures, and further evidence that they were immune complexes was obtained by demonstrating binding of guinea pig complement by immunohistochemical techniques. There was no single type of glomerulonephritis characteristic of hepatitis B surface antigen immune complexes. There was, however, a strikingly high frequency of antigen-positive patients among cases of glomerulonephritis of the membranous varieties, with and without cellular proliferation. This finding is in accord with reports of adult patients with hepatitis B antigenaemia which co-existed with glomerulonephritis and surface antigen immune complexes in glomeruli that displayed membranous lesions and moderately increased cellularity. Hepatitis B antigen immune complexes were also found in hyaline and fibrinoid arteriolar lesions in cases of endocapillary and extra-

capillary glomerulonephritis. The high incidence of hepatitis B antigen-positive cases among children with immune-deposit glomerulonephritis, which may be a local phenomenon in Poland, greatly exceeds that noted in other diseases or populations, except for polyarteritis nodosa, and it approaches that found in patients with chronic hepatitis. It is noted that the number of transfusions of plasma or whole blood given to patients with nephritis in Poland is significantly higher than elsewhere. Several case reports of patients with hepatitis B and glomerulonephritis have also been reported from other countries. The characteristic lesions in such patients have been a membrano-proliferative, membranous, endocapillary or extracapillary proliferative glomerulonephritis.

Gyorkey et al. (1975) obtained experimental evidence for the development of glomerulonephritis in three out of 15 non-human primates (ten baboons and five rhesus monkeys) inoculated with varying amounts of human plasma containing hepatitis B surface antigen. The lesions consisted of progressive focal glomerulonephritis with mesangial alterations which developed over a period of 4 to 10 months after inoculation. The most likely explanation is immune complex deposition. The possibility that the immune complex deposition may have resulted from an antibody response to human serum which was injected can be ruled out, since in rabbits injected with a single large dose of bovine serum albumin any renal lesions were reversible and undetectable by the fourth week after injection. In the primates the renal lesions were progressive and a temporal progression of these lesions was observed which was similar to that reported in the chronic bovine serum albumin–rabbit model.

POLYMYALGIA RHEUMATICA

Polymyalgia rheumatica is a disease of unknown aetiology which in some cases is linked to giant-cell arteritis. The acute onset suggests an infective process, but an organism has not been demonstrated. Possibly the disease is an immunological disorder triggered by a viral infection. Bacon et al. (1975) tested this hypothesis by following a group of patients with polymyalgia rheumatica serially, measuring the immunoglobulins and antibody titres to a range of viruses, as well as through various other tests. Hepatitis B surface antibody was detected in nine out of 12 patients tested before treatment. The surface antibody persisted for up to 6 months in four patients but reverted to negative in the other five patients. The surface antigen was not detected in any patient or control and no significant

elevation of antibody titre was detected to a panel of 12 other micro-organisms. Immunoglobulin levels were elevated prior to treatment in several patients, but with steroid therapy the IgG and IgA levels fell serially, although the IgM levels increased in six patients. The results suggested that hepatitis B may be an important trigger for this condition and that polymyalgia rheumatica may represent an abnormal immuno-logical response to infection in elderly patients. Muller-Schoop *et al.* (1977) also reported hepatitis B surface antibody in polymyalgia rheuma-tica. Plouvier *et al.* (1978) described a patient with polymyalgia rheumatica and abnormal liver function who did have hepatitis B surface antigen in the serum as well as on the internal lamina elastica of the temporal artery. These authors also considered that hepatitis B virus infection could cause the initial clinical and biological manifestations of polymyalgia rheumatica and that the virus may be immunopathogenic by virtue of its antigenic rather than its viral properties.

ESSENTIAL MIXED CRYOGLOBULINAEMIA

McIntosh *et al.* (1976) examined sera from patients with acute hepatitis B, persistent hepatitis B antigenaemia, acute and chronic hepatitis not associated with hepatitis B surface antigen, various liver diseases and sera from healthy individuals for the presence of cryoproteins. Cryoproteins were found in the sera of many patients with acute and chronic hepatitis associated with hepatitis B surface antigen and in persistent antigen carriers. The quantity of these cold insoluble precipitates was highest in acute hepatitis. The precipitates contained either the surface antigen, surface antibody or both, and immunoglobulins and occasionally com-plement and rheumatoid factor. The cryoproteins possessed biological properties of immune complexes and several of the patients had clinical features of acute or chronic type III hypersensitivity reaction.

Essential mixed cryoglobulinaemia, unrelated to a well-defined con-nective tissue disease, lymphoreticular neoplasia or obvious infectious process, is associated with widespread vasculitis and immune complex deposition which is believed to be the underlying mechanism. Levo *et al.* (1977) noted the reported high frequency of clinical or laboratory evidence of liver involvement and found, on examination, that 14 out of 19 (74%) cryoprecipitates obtained from patients with mixed cryoglobulinaemia were positive for hepatitis B surface antigen or surface antibody. Both the IgG and IgM fractions had surface antibody activity. It was concluded

that most of the cases should probably be classified as mixed cryo-globulinaemia secondary to viral infection, most commonly hepatitis B virus, but perhaps also with other as yet unrecognized viruses.

INFANTILE PAPULAR ACRODERMATITIS

Infantile papular acrodermatitis (Gianotti–Crosti syndrome) is a distinct clinical entity characterized by a non-itching erythematous papular skin eruption on the face and extremities, lymphadenopathy and hepatomegaly with abnormal liver function. This syndrome has been predominantly reported in Mediterranean countries, and a close association with hepatitis B surface antigen was first recognized by Gianotti (1973), who detected the antigen in each of 39 children with papular acrodermatitis. In 1976, Ishimaru *et al*. described an epidemic of this condition in a city in Japan. The surface antigen, subtype *ayw*, was found in most of the affected children when tested on the first day of the skin eruption, and the antigen persisted for up to 4 months in a little over half the children. An immune complex mechanism is implicated in the pathogenesis of this syndrome. In this context it should be noted that various skin rashes occur in about 5% of patients with acute viral hepatitis. Weiss *et al*. (1978) reported observations which were consistent with an immune complex deposition in the skin of a patient with acute hepatitis B after the maculopapular rash became purpuric.

THE DELTA ANTIGEN–ANTIBODY SYSTEM

During the examination by direct immunofluorescence of liver biopsies from patients with circulating hepatitis B surface antigen, it was noted that a serum containing core antibody stained not only core antigen, demonstrable by electron microscopy, but also nuclei which did not contain core particles (Rizzetto *et al*., 1977). Further investigation revealed that some antisera to the core antigen reacted with either one or other liver substrates associated with infection with hepatitis B virus. The reacting antigen is distinct from the surface antigen, the core antigen and *e* antigen, and is termed the delta antigen. This new antigen was detected by direct immunofluorescence in the liver cell nuclei of patients with chronic liver disease associated with hepatitis B surface antigen. The delta and core antigen determinants in liver cell nuclei appear to be mutually exclusive. Delta antibodies may be present or absent in sera containing

core antibody. This new antibody was found in the serum of persistent carriers of the surface antigen, with a higher prevalence in patients with liver damage. Crivelli *et al.* (1978) developed an immunofluorescence blocking test which allows the detection of anti-delta in serum by specific inhibition of the fluorescence produced by delta antisera conjugated with fluorescein isothiocyanate. Delta antibodies were found in 41 (19·4%) out of 211 sera containing the surface antigen, but not in any of 50 sera without detectable surface antigen.

The nature of the delta antigen is unknown at present.

AN ANTIBODY REACTING WITH DANE PARTICLES

Alberti *et al.* (1978) described the presence of antibodies reacting with specificities on the complete hepatitis B virus (the Dane particle). These antibodies did not correlate with anti-*e*, although it was considered possible that only one of the various determinants of the *e* antigen was involved. An important observation was that these precipitating antibodies may be relevant to the clearance of circulating hepatitis B virions and the termination of acute infection, and their absence in all but one of the patients with chronic active hepatitis might explain why the infection persists in such patients. These antibodies might also be of relevance in protective immunity, and vaccines prepared from the complete hepatitis B virus containing viral components other than just the surface antigen are being considered (Prince *et al.*, 1978).

13 | The carrier state

Hepatitis A is probably maintained by serial transfer by the intestinal-oral route since there does not appear to be a major human or animal reservoir of the virus. On the other hand, numerous seroepidemiological surveys, mostly on selected population groups, reveal that there is a large reservoir of persistent carriers of hepatitis B virus in the world estimated to number about 176 million, although even within each country considerable differences in prevalence exist between different ethnic and socio-economic groups. The association of the third form of viral hepatitis (non-A : non-B hepatitis) with blood transfusion, linked with the fact that this infection does not spread readily from person to person, implies a close resemblance to hepatitis B and the existence of an infectious carrier state.

HEPATITIS A

Human volunteer studies have shown that excretion of infective virus in faeces and a transient viraemia occur in patients during the third and fourth week of the incubation period of hepatitis A and during the acute phase of the illness. Volunteer studies at Willowbrook State School revealed that faeces were infective on day 25 of the incubation period and on days 1-8 after the onset of jaundice. Viraemia was demonstrated on

day 25 of the incubation period, 3–7 days before the onset of jaundice and 3 days after the onset of jaundice. Attempts to demonstrate infective virus in the faeces or in the blood at some other times were unsuccessful.

Transmission of human hepatitis A infection to chimpanzees (Maynard et al., 1975; Thornton et al., 1975; Dienstag et al., 1975) confirmed the presence of hepatitis A virus in faeces during the incubation period and early acute phase of illness. In a more comprehensive study Thornton et al. (1977) infected several chimpanzees with different strains of hepatitis A virus. Hepatitis A virus particles were found in faeces as early as 9 days after infection and generally by days 12 and 14. Excretion of virus continued for 9 to 19 days, and the maximum number of virus particles were found 17 and 19 days after inoculation. In a subsequent study, Bradley et al. (1977) found the virus in faeces of infected chimpanzees 14 and 15 days after inoculation, but cyclic excretion of virus was demonstrated both earlier and later during the incubation period.

The use of sensitive techniques such as radioimmunoassay and enzyme immunoassay for hepatitis A antigen revealed that the antigen may be detected in faeces as late as 14 days after maximum elevation of serum alanine transferase in one study (Hall et al., 1977), although the maximum numbers of virus particles were found during the incubation period 15 to 5 days before peak alanine transferase elevations.

Hepatitis A is not transmitted by blood and blood products and rarely, if ever, by the parenteral route (Szmuness et al., 1977; Papaevangelou et al., 1978), although this has been achieved by experimental transmission to human volunteers and to non-human primates.

On the basis of the above evidence and the development of specific hepatitis A antibody to a high titre during the late incubation period and early acute phase of the illness, persistent carriage of hepatitis A virus or persistent excretion of this virus in the faeces does not occur.

HEPATITIS B

Experimental transmission studies of hepatitis to human volunteers during World War II and extending into the early 1950s revealed the presence of viraemia, in the case of hepatitis type B, up to 87 days before the onset of illness (Neefe et al., 1944b; Paul et al., 1945; Havens, 1946). Evidence that a prolonged carrier state for as long as 5 years may result in some patients, with or without signs of liver disease, has been provided by Stokes et al. (1954), Neefe et al. (1954) and Murray et al. (1955a, b).

Murray *et al.* (1954) recorded the case history of a donor in whose blood hepatitis B virus was found 135 days after he donated blood which was subsequently incriminated as the cause of hepatitis in the recipient. The same donor later developed hepatitis himself. Viraemia was demonstrated once again, on this occasion 6 months later.

Zuckerman and Taylor (1969) detected hepatitis B surface antigen in the serum of a former blood donor PJG, who was identified as a carrier in 1951 (Stokes *et al.*, 1954). This donor was implicated in 1950 in three cases of homologous serum jaundice, one of whom died 73 days after transfusion. There was no suggestion in his past medical history that he had even suffered from hepatitis. Ten months after the first incident of infection occurred in a recipient of his blood, a sample from PJG was injected in the U.S.A. into five volunteers. At least one of the volunteers developed hepatitis with jaundice 59–62 days after intramuscular inoculation. Hepatitis B surface antigen was detected by immunodiffusion, counter-immunoelectrophoresis and electron microscopy in fresh serum collected from PJG in 1968, 1969, 1970 and 1971, and furthermore, the antigen was found by electron microscopy in the original 1950–1951 serum (MacCallum, 1972a). This is a well-documented example of apparent healthy carriage of hepatitis B antigen for over 20 years.

Giles *et al.* (1969) tested for hepatitis B surface antigen serial samples of serum obtained before and up to 200 days after experimental infection of volunteer children with serum containing the MS-2 strain of type B hepatitis. Surface antigen was detected in all patients after infection with MS-2, frequently before there was any clinical evidence of infection. In approximately half the patients the antigen persisted in the serum for 200 days and subsequently for at least 3 years after infection.

These observations confirmed the long-held epidemiological and clinical findings that a prolonged carrier state of hepatitis B virus may exist with persistence of the virus in the blood. With the development of sensitive laboratory techniques for the detection of markers of infection with hepatitis B it is now estimated that there are some 176 million carriers in the world today.

On the basis of longitudinal studies of patients with hepatitis B, the persistent carrier state has been defined as the presence of hepatitis B surface antigen for more than 6 months (WHO Expert Committee on Viral Hepatitis, 1977). Such a carrier state may be associated with liver damage.

Extensive surveys in many parts of the world have shown that the

prevalence of hepatitis B surface antigen in apparently healthy persons varies from 0·1 to 20%. Europe may be roughly divided into three regions, with a low prevalence of about 0·1% or less in northern countries, 0·1 to 3% in some central and eastern European countries, and about 5% and even higher in some countries bordering the Mediterranean. The prevalence in North America and Australia is 0·1% or less, 3–5% or higher in Asia and 15% in several tropical countries. It should be noted, however, that the global distribution of hepatitis B surface antigen is by no means complete and furthermore, standardized techniques and reagents have not been uniformly used.

A number of risk factors have been identified in relation to the development of the carrier state. It is more common in males, more likely to follow infections acquired in childhood than those acquired in adult life, and more likely to occur in patients with natural or acquired immune deficiencies.

In countries in which infection with hepatitis B virus is relatively uncommon, the highest prevalence of the surface antigen is found in the 20–40 year age group. The prevalence of surface antibody increases steadily with age. In countries where infection with hepatitis B virus is common, the highest prevalence of the surface antigen is observed in children 4–8 years old, with declining rates among older age groups. The decline in antigen carriage rates with age suggests that the carrier state is not invariably lifelong. Hepatitis B *e* antigen has been found more commonly in young than in adult carriers, while the prevalence of anti-*e* appears to increase with age (Ohbayashi *et al.*, 1976). These findings suggest that young carriers may be the most infective.

LIVER DISEASE IN CARRIERS

Various serological surveys and the routine screening of donor blood have identified many asymptomatic carriers of hepatitis B surface antigen. A varying proportion of such carriers has been found on investigation to have abnormalities in the liver ranging in severity from minor changes in the nucleus of the cell to severe chronic active hepatitis and cirrhosis. The subject has been extensively reviewed by Zuckerman (1975b), Klatskin (1975) and others. Two studies illustrate the problem particularly well. Woolf *et al.* (1974) investigated 34 blood donors in the Manchester area in England, who were found to be persistent carriers of the surface antigen. Histological abnormalities were found in 31 of the 34 donors, including

one carrier with cirrhosis, three with chronic aggressive hepatitis and 11 with chronic persistent hepatitis. In 13 biopsies there were focal areas of necrosis in the liver parenchyma. These varied in size in different individuals. Eighteen of the 34 donors had abnormalities of routine biochemical tests of liver function. These included seven of the 11 donors with chronic persistent hepatitis, the donor with cirrhosis and all three with chronic aggressive hepatitis. Seven of the 17 donors with focal necrosis also had abnormalities of liver function. Bromsulphthalein retention tests were abnormal in 15 donors, including the donor with cirrhosis, six of those with chronic persistent hepatitis and three with chronic aggressive hepatitis. None of the donors with normal liver histology showed any abnormality by the routine tests.

Tapp and Jones (1977) stained 48 biopsies obtained from the liver of 33 asymptomatic blood donor carriers of the surface antigen for the surface and core antigens by the immunoperoxidase technique and with orcein. A focal cytoplasmic distribution of the surface antigen predominated in the hepatocytes of carriers showing the histological lesions of chronic aggressive hepatitis while a diffuse distribution of the surface antigen was seen more frequently in those showing minor or no histological abnormalities. In general there was an inverse relationship between the amount of surface antigen in the cytoplasm and the activity of the disease in the liver. All the biopsies that were positive for core antigen in the nuclei of hepatocytes showed either chronic aggressive or chronic persistent hepatitis. In carriers who had more than one liver biopsy, the presence of stainable surface antigen, irrespective of the initial histological diagnosis, was associated with an increased likelihood of progression of the histological lesion. These and other studies indicate that the term "healthy carrier" may be a misnomer and that asymptomatic carriers of hepatitis B surface antigen should remain under medical supervision.

SEX DIFFERENCES IN THE CARRIER STATE

An excess of males with hepatitis B surface antigen was noted in early extensive family studies of the distribution of Australia antigen carried out in the islands of Cebu in the Philippines and in Bougainville, New Guinea (Blumberg et al., 1968). A higher incidence of the surface antigen was also found among male patients with G-trisomy or Down's syndrome resident in institutions, among blood donors, and among patients with acute hepatitis B. London et al. (1977) also reported that males were more

likely to become persistent carriers when treated by maintenance haemo-dialysis, and females were more likely to develop surface antibody.

Robertson and Sheard (1973) reported that the sex ratio at birth in a small town in Lincolnshire in England was disturbed in 1969. There were 43 live female births but only 21 live male births and one male stillbirth. There was no increase in the incidence of spontaneous abortions in the town. It was pointed out that the ratio of boys to girls could be affected by any factor which impaired the viability of sperms bearing the Y chromo-some, by factors which gave some advantage in penetration of mucus or ovum to X-bearing sperm, or after conception, by factors which selectively impaired the viability or survival of the male embryo. A striking excess of female births occurred twice before in the same town in 1955 and 1967. The most notable feature of 1967 in the town was a severe outbreak of viral hepatitis and Robertson and Sheard considered that subclinical and anicteric hepatitis may have persisted in the town until 1970. An excess of males among siblings of carriers of hepatitis B surface antigen in a Melanesian population on the island of Santa Cruz in the British Solomon Islands has also been reported and it has been suggested that this alteration in the sex ratio might be the result of selection by an infectious disease.

Hesser et al. (1976) reported that matings with a parent in whom the surface antigen was present resulted in an increased sex ratio among males (64%) in a Greek population compared with matings of negative parents (53% males). In Melanesian populations, however, the sex ratio was decreased when the mother was positive for surface antigen and increased when the father was positive. Thus the presence of the surface antigen is associated with alterations in the live birth sex ratio, and altered sex ratios in turn affect population reproduction rates.

The interactions between hepatitis B virus and sex have been studied further. London et al. (1977) reported that among patients receiving treatment in a haemodialysis clinic, males had a 68% chance of remaining persistent carriers of the surface antigen once infected with hepatitis B virus, whereas females had only a 33% chance. Conversely, females had a 55% chance of developing surface antibody once infected, whereas males had only a 30% chance. In addition, the response of renal graft recipients to infection with hepatitis B virus before transplantation and the sex of their kidney donors was related to the duration of graft survival (London et al., 1977). Kidneys from HLA non-identical male donors which were transplanted into male or female patients with surface antibody survived only a few months. However, survival of grafts from male donors was

significantly longer in both uninfected patients and in persistent surface antigen carriers. There were no differences in graft survival among the relatively few patients who received grafts from female donors. The reason why patients with surface antibody have accelerated rejection of kidney transplants is not known. This may be related to an early recognition of HLA antigens, but the observation that the greatest risk of graft rejection occurred when the recipient had surface antibody and the kidney donor was a male is not explained by the hypothesis. Another hypothesis is that Y-linked histocompatibility antigens (H–Y antigen) influenced the host response to both the surface antigen and the HLA antigens.

Drew *et al.* (1978), as a result of an investigation of the response to infection with hepatitis B virus and the sex ratio in a Greek village together with the above observations, put forward the hypothesis that there is cross-reactivity between the surface antigen and a male-associated antigen. If hepatitis B surface antigen cross-reacts with a male-associated antigen, males would be more likely to recognize the surface antigen as "self" and therefore would persistently carry the antigen. Females, however, would be more likely to recognize the surface antigen as "foreign" and produce surface antibody. In renal transplant patients, tolerance to the surface antigen would result in relative tolerance of male tissues, whereas surface antibody in the recipient would react with male antigens or renal allografts from male donors leading to early rejection of the grafts. Similarly, it is speculated that tolerance to the surface antigen in pregnant women (i.e. in carriers) would result in lack of sensitization against male tissues, and therefore good survival of male foetuses. Surface antibody, on the other hand, could react with male antigens and perhaps hinder fertilization by sperm bearing a Y chromosome or increase the probability of spontaneous abortion of male foetuses. Male carriers of surface antigen would have the antigen in their semen which could perhaps protect Y-bearing sperm from surface antibody in the reproductive tract of their spouses. The effect of foetal loss in antigen-carrying women could be explained by replication of hepatitis B virus in male foetuses, and this could result in a greater loss of male than female embryos. In summary, in either parent, surface antigen was found to be associated with a high sex ratio (of males to females) in live births, whereas surface antibody in mothers was associated with a low sex ratio. Female loss in antigen-positive mothers was also related to a lower sex ratio. Further studies of this important observation and hypotheses are clearly required.

In this context it should be noted that sex differences in susceptibility to infections have been investigated previously. For example, in the case of bacterial meningitis at all ages and bacterial septicaemia in children under 15 years of age a significant preponderance of males was found and it was most marked in infancy. This is also the period during which infections by Gram-negative enterobacteria outnumber those by Gram-positive bacteria and this difference was found to affect males more than females. The sex differences in susceptibility to these infections were postulated to be consistent with a genetic hypothesis concerning a gene locus on the X chromosome of human beings, which is involved in the synthesis of immunoglobulins. Small differences in amounts or rates of synthesis of immunoglobulins might be responsible for a slightly greater susceptibility to infection among some members of one sex. In general, greater resistance to infectious diseases might be expected in that sex for which heterozygosity is possible, but Washburn *et al.* (1965) concluded that more direct evidence for this hypothesis must be obtained from comparative studies of the immune mechanism in the two sexes. Rhodes *et al.* (1969) investigated 28 women with an additional X chromosome (XXX) and an equal number of normal men and women matched for age. The mean serum levels of IgM were found to be highest in the XXX group, intermediate in normal women and lowest in men. The IgG levels were also significantly higher in the XXX females than in either normal men or women. These results and the fact that there is an X-linked basis for the most common form of congenital agammaglobulinaemia support the suggestion that a gene locus on the X chromosome has at least some regulatory effect on antibody production in humans. Michaels and Rogers (1971) found that the serum levels of *Escherichia coli* haemagglutinins were slightly higher in 120 females in hospital aged 6 to 14 months. In addition, in each of ten field trials, involving a total of over 15 000 children, the antibody response to rubella vaccine in females exceeded slightly the antibody response in males.

GENETIC SUSCEPTIBILITY TO HEPATITIS B

Blumberg *et al.* (1969) put forward a genetic hypothesis to explain the geographical variation in the prevalence of apparently healthy carriers of hepatitis B surface antigen. Examples of inherited susceptibility to infection have been described in several animal species including *Homo sapiens* and it is recognized, of course, that many other factors in the

environment such as sex, age at exposure, state of nutrition, immuno-
logical factors, socio-economic circumstances and so on also affect sus-
ceptibility and resistance to infection. The discovery of hepatitis B
surface antigen resulted from the interest of Blumberg and his associates
in inherited polymorphisms. Family studies carried out in the islands of
Cebu and Bougainville in the Pacific, and on a more limited scale elsewhere,
appeared to be consistent with the hypothesis that a gene designated Au^1
is responsible for persistence of the surface antigen once infection with
hepatitis B virus has occurred. This gene is considered to be common in
tropical areas but rare in temperate zones. Individuals homozygous for
this gene (phenotype Au^1/Au^1) would have persistent antigen without
overt manifestation of hepatitis although they may remain as carriers of
the associated infectious agent, whereas the heterozygotes (phenotype
Au^1/Au^0) and individuals without the gene (Au^0/Au^0) would not remain as
carriers. Persistence of the surface antigen in certain other diseases such as
leukaemia, lepromatous leprosy and Down's syndrome is believed to be of
a different nature, namely an inherited susceptibility to infection not only
with hepatitis B but with the agents responsible for these other diseases.
This could explain the association of the antigen with other diseases under
circumstances in which the individuals with the susceptibility gene were
exposed to hepatitis B virus; and absence of the association when exposure
did not occur.

A similar conclusion on the presence of an autosomal recessive locus was
reached in studies in Sardinia although with some distinct qualifications.
Firstly, there was a sharp decline in the carrier rate of the antigen after
the age of 20, an observation which has also been reported from many
other high prevalence areas. Secondly, all of the reported matings,
excepting one family, were between parents where only one parent was
antigen-positive or where both parents were negative. In the family in
which the antigen was detected in both parents, only two of the seven
children were also positive. This finding on its own would rule out a
simple hypothesis of recessive inheritance with complete penetration of
the homozygous recessive genotype.

Other critical matings which also fail to support the postulated in-
herited susceptibility to persistence of hepatitis B surface antigen have
been reported. A pedigree with antigen-carrying parents and their progeny
of three children were tested for hepatitis B surface antigen and surface
antibody by several methods including sensitive techniques, such as radio-
immunoassay and passive haemagglutination. The antigen was not found

by repeated testing of the serum of the three children. However, surface antibody was detected in the serum of the eldest son, aged 10 years, providing unequivocal evidence of past exposure to the antigen resulting in a normal immune response to infection. This is crucial evidence against susceptibility to the persistent carrier state inherited as a simple autosomal recessive trait (Vyas, 1974). Stevens and Beasley (1976) found a large number of families in Taiwan with two antigen carrier parents, during a study of vertical transmission of hepatitis B infection. Data from these families, compared to those with a carrier mother and antibody-positive father, were incompatible with an autosomal recessive mode of inheritance. The development of persistent antigenaemia in the newborn children or its existence in their siblings was independent of whether the father was an antigen carrier or antibody-positive, but it appeared to be dependent on the time of infection, the titre of the antigen (virus) and the presence of e antigen. Mazzur (1976), as a result of investigation of a large family and genetic studies on the distribution of hepatitis B surface antigen and surface antibody in Santa Cruz Island in the Solomon Islands also reached a similar conclusion. Additional evidence against the genetic hypothesis was provided by a family in which both parents were negative for the surface antigen and all their four children were carriers.

Although there are many reports of familial clustering, genetic factors are not necessarily involved, since vertical transmission appears to occur and perinatal transmission from mother to child also takes place. The alternative hypothesis to the genetic factor is, of course, that familial clustering may be entirely a function of an increased opportunity for environmental exposure to the virus. Furthermore, it stands to reason that if a parent is excreting the virus, this will augment the chance that any of the offspring may acquire it, particularly if the mother is the transmitter.

Another aspect of the genetic basis of susceptibility to hepatitis B is implicit in the finding that the frequency of certain genetic markers on the heavy chains of immunoglobulin G (Gm types) was always greater in multiply transfused Italian patients with thalassaemia who were persistent carriers of hepatitis B surface antigen than in those with hepatitis B surface antibody (Blumberg et al., 1972). With respect to Gm types, individuals who are heterozygous for Gm factors are less likely to encounter Gm types different from their own and are therefore less likely to develop anti-Gm. Such persons when infected with hepatitis B virus, which incorporates certain host components in its protein coat, would be

likely to develop persistent antigenaemia and minimal liver damage. On the other hand, individuals who are homozygous for Gm types (i.e. they have fewer Gm types) are more likely to encounter Gm types different from their own and thus develop antibodies to them. Such persons, when infected with hepatitis B virus, would be more likely to have an acute infection and an increased probability of developing hepatitis B antibody. It was also considered that the same (or similar) agent which leads to the development of antibody to hepatitis B surface antigen may lead to the development of anti-Gm in an individual exposed to it either by transfusion or infection. A corollary of this is that the "SNagg" antibodies (agglutinins in normal sera) and "Ragg" antibodies (rheumatoid factor) are due to infection with the hepatitis B virus, or an agent similar to it. Schanfield et al. (1973) examined for Gm allotypic marker samples from blood donors submitted routinely for testing for hepatitis B surface antigen in San Francisco and Minneapolis. The frequency of the various Gm phenotypes did not vary significantly among the Caucasian donors investigated according to the presence or absence of hepatitis B surface antigen or its antibody. Similarly, there was no significant excess of anti-Gm among individuals with hepatitis B surface antibody when compared with those with persistent hepatitis B antigenaemia. These findings are not in accord, at least in a normal adult population, with the hypothesis of a direct association between the polymorphisms of the Gm types and hepatitis B surface antigen. In the course of a large multicentre study, Schanfield et al. (1975) found that geographical variations in the prevalence of hepatitis B infection were associated with an increased opportunity of exposure to this virus in patients with homozygous β-thalassaemia in Cyprus, Greece, Sardinia and the United Kingdom. No association was found between the Gm phenotype of the patients and susceptibility to hepatitis B virus using the surface antigen as a marker, or the ability to make antibody to it. It was concluded that variation in the distribution of Gm haplotypes does not appear to be a feasible explanation for variation in the frequency of surface antigen or surface antibody in a series of transfused populations.

THE HISTOCOMPATIBILITY LOCI (HLA)

Mackay and Morris (1972) summarized the relevance of the study of the distribution of HLA antigens, the major histocompatibility system of humans, in the investigation of genetic determinants of immunopathogenic

diseases. The following were listed: the known link between the genetic
control of immune responses to synthetic antigens of limited hetero-
geneity or to limiting doses of native proteins and the major histo-
compatibility system in the mouse and the guinea pig; the suggested role
of histocompatibility systems in the generation of antibody diversity; the
association between the major histocompatibility system in the mouse
(H2) and susceptibility to viral leukaemia; and the association between
HLA antigens and Hodgkin's disease and systemic lupus erythematosus.
Mackay and Morris found that the frequency of HLA-A1 and HLA-A8
(60% and 68%, respectively) in 37 patients with active chronic hepatitis
was significantly increased in comparison to that found in patients with
other liver diseases (29% and 25%) and in normal Caucasians residing in
Melbourne (31% and 18%). This was especially evident in the prototype
group of young women with lupoid hepatitis. A hypothetical influence of
genes expressing HLA-A1, HLA-A8 on the proliferative or tolerance re-
sponse of immunocytes was suggested as a mechanism which could account
for the linkage disequilibrium between these antigens, and also help in
the interpretation of the inherited component in autoimmune disease.

A high frequency of HLA-A1 and HLA-A8 has been reported from
several centres in patients with chronic active hepatitis not associated with
hepatitis B surface antigen (see, for example, Galbraith et al., 1974;
Lindberg et al., 1975). It would thus appear that genetic differences
expressed through histocompatibility antigens could be a factor accounting
for differences in racial prevalence of disease in general and for the
autoimmune type of chronic active hepatitis in particular.

Cold reacting complement-dependent lymphocytotoxins (CoCoCy), a
new type of autoantibody, was found in low titre in 48·1% of the patients
with chronic aggressive hepatitis and in 13·7% of the patients with
chronic persistent hepatitis. The frequency of the antibody in controls
was 6%. The role of this antibody in chronic liver disease is not yet clear,
but a reciprocal relationship was found between CoCoCy and HLA-
W18 and hepatitis B surface antigen in chronic aggressive hepatitis.
However, CoCoCy did not exhibit any HLA specificity.

The relationship between HL-A antigens and hepatitis B surface
antigen has also been studied. An association has been reported between
the persistent carrier state of the surface antigen and HLA-A3 and W19
at the LA-locus in a random Belgian population. An association was also
established by Boettcher et al. (1975) between an HLA antigen and the
presence of hepatitis B surface antigen in a group of Australian aborigines.

There was a statistically significant deficiency of W15 among carriers of the antigen and this was explained in terms of linkage disequilibrium between W15 immune and an immune response gene involved in clearing the surface antigen from the body. While it was pointed out that the hypothesis of becoming a carrier being largely genetically determined was an oversimplification, it was accepted that the potential of becoming a carrier was a recessive condition controlled by a single autosomal gene. It was proposed that if the antigen W15 was a (hepatitis B) virus receptor on cells, and it was disadvantageous to have viruses located on these receptors of certain cells, it would be of selective advantage to also possess the immune response gene responsible for clearing viruses from the receptors. Under these circumstances, an association of W15 and the hepatitis B surface antigen immune response gene would be of selective advantage.

Vladutiu (1978) suggested that the association between HLA antigens and persistent infection with hepatitis B virus and indeed with other infections might reflect the dependence of T lymphocyte cytotoxicity on particular histocompatibility antigens. Individuals heterozygous for HLA antigens should eliminate viruses more efficiently and hence an increased number of homozygotes for HLA antigens might be expected in carriers of hepatitis B virus. This has been observed in patients with chronic hepatitis by Page et al. (1975). As noted above, an increased frequency of HLA-A3 and A19 has been found in carriers by Vermlyen et al. (1972), and in antigen-positive patients with chronic active hepatitis there was an increased prevalence of HLA-BW35 (Penner et al., 1977). On the other hand, a deficiency in the frequency of HLA-A1 and B8 phenotypes was reported in patients with uraemia who were able to eliminate the surface antigen (Descamps et al., 1977). An association between HLA-BW15 and transient hepatitis B surface antigenaemia, and HLA-BW17 and persistent antigenaemia, has also been noted (Hillis et al., 1977).

A state of transient antigenaemia suggests that immune defence mechanisms are increased and clear the virus, while persistent antigenaemia suggests an impairment of the immune system. Increased incidence of particular HLA antigens accompanying transient antigenaemia and deficiency of some HLA antigens in persistent carriers suggest that hepatitis B virus preferentially influences some HLA genes or gene products allowing an augmented T cell lysis of the infected cells. This hypothesis is supported by the finding of an increased frequency of HLA-A1 and B8 in patients who eliminated the surface antigen whereas these HLA antigens were lacking in all patients who did not eliminate

the virus (Galbraith *et al.*, 1974; Eddleston *et al.*, 1976). Similarly, HLA-BW15 was deficient in asymptomatic carriers and increased in individuals with transient antigenaemia (Hillis *et al.*, 1977). Vladutiu (1978) also considered that hepatitis B virus decreased the number of HLA antigenic sites on the membranes of hepatocytes, preventing the recognition of infected cells by the cytotoxic T lymphocytes. The increase of some HLA antigens, such as HLA-A3 and A19 seen in asymptomatic carriers, suggested that these antigens were not altered by or not immunologically associated with hepatitis B virus. More information is required to test this interesting hypothesis.

MANAGEMENT OF THE ASYMPTOMATIC CARRIER STATE OF HEPATITIS B

This is a complex and vexed issue with considerable personal, social and economic implications. Guidance on management requires consideration of individual circumstances and general recommendations are found in the World Health Organization Reports (1973, 1975, 1977), in the revised statement by the Committee on Viral Hepatitis of the Division of Medical Sciences, National Research Council of the United States of America (1974), and the second Report of the Advisory Group on hepatitis and the treatment of chronic renal failure (Chairman: Lord Rosenheim, United Kingdom, 1972). The subject is also reviewed, amongst others, by Chalmers and Alter (1971), Alter *et al.* (1975) and Blumberg (1976).

The following is the revised statement by the Committee on Viral Hepatitis of the Division of Medical Sciences, National Research Council of the United States of America (1974):

A clearer definition of the significance of viral hepatitis type B as a clinical and public health problem has arisen from the discovery, development and widespread application of various serological tests for the presence of an antigen—hepatitis B antigen—that is associated with the disease. The demonstration of the antigen in the blood of a patient or of an apparently healthy person raises questions not only of the presence of active liver disease, but also of the potential risk of transmission of the infection to others. It is now recognised that, in addition to the well-established parenteral mode of transmission, viral hepatitis type B can be transmitted by other means.

On the basis of information acquired from clinical and epidemiological

studies and from antigen testing programme, the Committee on Viral Hepatitis finds that:

(1) A confirmed positive test for antigen is indicative of acute or chronic viral hepatitis type B or of an asymptomatic carrier state.

(2) The presence of the antigen in the blood of a patient with acute viral hepatitis type B is usually transient. If it persists for more than 3 months after the onset of illness, the person is likely to become a chronic carrier of the antigen.

(3) A chronic carrier of the antigen may or may not have demonstrable evidence of related liver disease.

(4) The occurrence of acute hepatitis type B or an asymptomatic carrier state during pregnancy or even during the first two months post-partum is frequently associated with later infection in the new-born infant.

(5) There is clear evidence that carriers should be prohibited from donating blood for transfusion.

(6) Although the infectiousness of patients with antigen-positive hepatitis apparently diminishes when the antigen is no longer demonstrable in the blood, they are currently not accepted as blood donors.

(7) There is insufficient knowledge of the extent to which chronic carriers can transmit hepatitis type B by nonparenteral routes. However, close contacts of some categories of chronic carriers, such as renal dialysis patients, are at increased risk for hepatitis type B infection.

(8) With respect to risk of transmission to others, there is no indication at this time that routine antigen testing of any specific professional or occupational group should be required.

(9) Standard Human Immune Serum Globulin (ISG)* is of no demonstrable value in the treatment of carriers.

(10) There is insufficient evidence on which to recommend the use of standard ISG* for prophylaxis among contacts of hepatitis B patients or carriers. Studies of the possible prophylactic effect of hepatitis B hyperimmune serum globulin are currently in progress.

The Committee recommends that:

(1) Persons found to have a positive antigen test in the course of diagnostic studies, blood-donor testing, or testing after known exposure to infection with hepatitis type B be so informed and the test be repeated promptly; and persons with a confirmed positive test be evaluated for the presence of liver disease and followed to determine whether the antigen persists;

* This term refers to immunoglobulin prepared from large pools of plasma.

(2) Persons with antigen-positive hepatitis be considered infectious and control measures be taken with respect to potentially infectious materials, such as blood and blood-contaminated secretion;

(3) Women found to have hepatitis during pregnancy or during the first two months post-partum be tested for hepatitis B antigen and their infants be tested for hepatitis B antigen at monthly intervals for at least 6 months;

(4) Testing for hepatitis B antigen be required of all blood donors;

(5) Until more complete knowledge of the significance of the antigen carrier state is acquired, particularly as to its relation to communicability, only routine precautions, such as those applying to percutaneous routes of potential transmission, be initiated;

(6) The effort to obtain more accurate and complete reporting of hepatitis cases—on the basis of serological test results as well as epidemiological characteristics—be intensified to improve surveillance on a national basis.

A particularly difficult issue has arisen concerning carriers of hepatitis B surface antigen belonging to the medical or other professions in close contact with the general population. The WHO Expert Committee on Viral Hepatitis (1977) concurred with the recommendations made to WHO in 1973 and 1975 that:

at present there is no evidence that carriers of HBsAg belonging to the medical or other professions in close contact with the general population routinely present a hazard, provided that they take general hygienic precautions in their professional activities. Studies of their contacts should continue to determine under what specific conditions of transmission of infection may occur.

There is general agreement, however, with the recommendation of the Advisory Group on hepatitis and the treatment of chronic renal failure in 1972 (Chairman: Lord Rosenheim), that all staff should be screened before working in the main unit caring for patients in renal failure (dialysis and transplantation units) and not accepted if hepatitis B (surface) antigen is detected. Staff health should be monitored and when members are unwell they must seek immediate medical attention (see also World Health Organization Report, 1973). The WHO Expert Committee on Viral Hepatitis (1977) recommended the following measure, in addition to other precautions, to be employed in haemodialysis and oncology units: continuous screening of patients and staff for hepatitis B surface antigen from time of entry to the units, and the avoidance of contact between hepatitis B surface antigen-positive staff and susceptible patients.

14

Maternal transmission of hepatitis B and neonatal infection

The effects of viral hepatitis on the course of pregnancy and on the foetus have been studied since the report by Saint-Vel in 1862 on an epidemic of severe hepatitis in pregnant women in Martinique. The early literature contains scanty and incomplete data and many divergent views. The possibility of transplacental transmission of hepatitis virus does not appear to have been seriously considered until 1951 when Stokes et al. reported a series of 12 infants in whom hepatitis developed during the first 2 months of life. Six of the infants died, but although the pathological changes in the liver were similar in a number of respects to those found in viral hepatitis, there were some added unusual features. In 1954 Stokes et al. presented evidence of "silent carriage" of hepatitis B virus and their paper is of considerable relevance to the problem of maternal transmission of this infection. One of the carriers described by Stokes et al. was known to have transmitted hepatitis to at least four out of 12 recipients of his blood over a period of 3 years. His blood was also administered to four male volunteers, one of whom developed hepatitis. The second carrier was the mother of an infant who developed hepatitis at the age of 2 months. Volunteers inoculated with serum from both the mother and her infant developed hepatitis. A third carrier was known to have transmitted hepatitis to four recipients of his blood, including one volunteer.

The results of many investigations on vertical transmission of hepatitis B using relatively insensitive techniques for detecting hepatitis B surface antigen in maternal blood, cord blood and breast milk suggested that transplacental transmission of hepatitis B virus was rare and perinatal hepatitis B infection was uncommon (reviewed by Zuckerman, 1975c). Although reports on individual case histories provided evidence for maternal transmission of hepatitis B and the recognition of familial clustering was suggestive, it was the careful studies of Schweitzer et al. (1972, 1973a, b) which demonstrated that vertical transmission was frequent when the mother suffered from acute hepatitis B within 2 to 3 months of delivery. Other reports of prospective investigations conducted in the United States and Europe (summarized by Fawaz et al., 1975) revealed that when the mother has acute hepatitis B late in pregnancy or during the early post-partum period, approximately two-thirds of the offspring may acquire the surface antigen within 1–6 months. On the other hand, if maternal hepatitis occurred early in pregnancy, the surface antigen was detected much less frequently in the infant, and a similar relatively low frequency of transmission occurs when the mother is an asymptomatic carrier of the surface antigen. Stevens et al. (1975) examined serum specimens from mothers in Taiwan using complement-fixation and radioimmunoassay for the surface antigen. Antigenaemia developed in 63 out of 158 babies born to carrier mothers, and 51 of the antigenaemic babies became antigen-positive within the first 6 months of life. Three interrelated factors were found to increase the risk that the infant would acquire the surface antigen: a high titre of the surface antigen in the maternal circulation, presence of the antigen in the umbilical cord blood and antigenaemia in siblings. It was suggested that if babies remain antigenaemic into adulthood, vertical transmission could account for a large proportion of carriers in high prevalence areas of hepatitis B infection. Okada et al. (1976) tested serum samples from 23 pregnant asymptomatic carriers of hepatitis B surface antigen in Japan and their babies. Using the immunodiffusion technique the e antigen was found in the serum of ten of the mothers and anti-e in seven. All ten babies born to mothers with e antigen acquired persistent hepatitis B surface antigenaemia. In sharp contrast, surface antigen was not detected in seven babies born to mothers with circulating anti-e. Two of the remaining six babies acquired the surface antigen. Interestingly enough, all ten elder siblings of the newborn infants to mothers with e antigen were found to be asymptomatic surface antigen carriers, but the surface antigen was not

found in the three elder siblings of the infants born to the mothers with anti-*e*. From the results of the other serological tests carried out for markers of hepatitis B it was apparent that except for *e* antigen and anti-*e*, there were no remarkable differences in the titre of surface antigen, the titre of core antibody or in the subtypes of the antigen. Hepatitis B surface antigen was not detected in the serum of any of the fathers.

Gerety and Schweitzer (1977) reported that 12 of 18 infants born to 18 women who developed acute hepatitis B later during their pregnancy or early in the post-partum period became persistently infected with hepatitis B virus. Five of the 14 children born to 12 persistent carriers of hepatitis B surface antigen became infected with hepatitis B virus and developed persistent antigenaemia. Again it was found that the presence of the *e* antigen in the mother correlated with the development of chronic hepatitis B infection. The *e* antigen was also found in the circulation of five infants born to *e* antigen-negative mothers (but this merely reflects the relative insensitivity of methods of testing for *e* and anti-*e*). Hepatitis B virus was also transmitted from two mothers with *e* antibody, indicating that this antibody is not necessarily related to non-infectivity. Similar findings were reported by Chaudhuri *et al.* (1977).

It appears, therefore, that the rate of perinatal transmission of hepatitis B is variable, although reports from areas of the world with a high prevalence of carriers indicated transmission to 40–52% of neonates. A survey of 4452 pregnant women in the greater Paris area carried out by Dupuy *et al.* (1978) revealed 28 asymptomatic persistent carriers of hepatitis B surface antigen. Seventeen mother–infant pairs were studied at delivery and followed up. Core antibody was present in the serum of all these infants at birth and subsequently the titre decreased progressively, indicating passive transfer of this antibody. The surface antigen was not detected in any of the infants at birth, but eight became positive after an average time of 48 days. Five of these infants had mild clinical hepatitis, and the antigen was cleared from seven out of eight infants. One infant became a persistent carrier. These results differ from previous published reports from low hepatitis B prevalence areas, particularly in terms of the apparent high transmission rates from chronic carriers and low rate of subsequent carriage among the infants. However, the ethnic group of the mothers was not stated and this may be important since other studies have revealed a very low rate of transmission from Caucasian mothers. This is well illustrated by the survey conducted by Derso *et al.* (1978). Antenatal screening in the West Midlands of Britain for hepatitis B surface antigen

by counter-immunoelectrophoresis and later by reverse passive haem-
agglutination identified a prevalence rate of antigen of about one in 850.
About half of the infants of 297 carrier mothers had the surface antigen
in the cord blood, but of 122 infants followed up for over 3 months, only
17 (14%) were still positive for the antigen. Surface antigen was detected
by solid-phase radioimmunoassay in 101 (46%) of 220 cord-blood speci-
mens and in 44 (52%) of 85 breast milk samples. Complete virus particles
were identified by immune electron microscopy in two out of 16 samples
of cord blood examined. The most interesting observation was the marked
difference in the rate of vertical transmission in different ethnic groups.
The Chinese babies were a distinct group with a transmission rate of 64%.
The Afro-Caribbean mothers also transmitted the carrier state to their
infants, four out of 13 children becoming carriers. The transmission rate
in the Asian group, which was the largest, was only 8%. Of 39 babies of
European mothers, none became antigen-positive. Finally, the role of
breast feeding in mother-to-infant transmission of hepatitis B surface
antigen was found to be unimportant.

CONTROL OF HEPATITIS B INFECTION IN THE NEWBORN

Passive immunization

The role of passive immunization with high-titre hepatitis B immuno-
globulin remains unestablished and its protective effect is not proven
(Fawaz *et al.*, 1975; Cossart and Cohen, 1976), but in general the number
of infants treated is very small. However, Tamburro and Leevy (1973)
noted that none of 30 newborn infants of hepatitis B surface antibody-
positive mothers with detectable antibody in the cord blood developed
hepatitis during observation for 3 months to 2 years, despite living in an
environment with high prevalence of narcotic drug addiction and hepatitis
B. The data were interpreted as evidence that transplacental transfer of
hepatitis B surface antibody provides passive immunity to hepatitis B.
This, of course, would be difficult to attain by the administration of
hepatitis B immunoglobulin to antigen carrier mother, but the observa-
tions provide a rationale for passive immunization in the newborn to a
carrier mother. Indeed, Kohler *et al.* (1974a) reported that five out of six
infants born to mothers who contracted hepatitis B during pregnancy
subsequently developed evidence of infection with hepatitis B virus
within 5 to 12 weeks of birth, whereas four such babies who were treated

with immunoglobulin or plasma containing hepatitis B surface antibody remained antigen-negative for 4 to 14 months. Mollica et al. (1977) reported the case history of an asymptomatic woman carrier of hepatitis B surface antigen who had six children. Four of the children developed jaundice during the neonatal period, had symptoms compatible with hepatitis and died between the ages of 38 and 75 days. A fifth child was an asymptomatic carrier of the surface antigen. The sixth child was admitted to hospital with clinical hepatitis at the age of 3 months, and the surface antigen was detected in his serum. After treatment with a corticosteroid preparation he was infused on seven occasions with plasma containing a high titre of hepatitis B surface antibody. He made a slow recovery and antigenaemia disappeared. It was considered that treatment with hepatitis B surface antibody may have contributed to his recovery, although other attempts to use immune plasma for the treatment of neonatal hepatitis have been unsuccessful (Dupuy et al., 1975; Fawaz et al., 1975). Dosik and Jhaveri (1978) described a single case of prevention of neonatal hepatitis B in a family in which the first child died from this infection at the age of 3 months. The mother was a persistent carrier of the surface antigen but e antigen was not detectable. The second infant was treated with hepatitis B immunoglobulin on the day of birth and every 5 weeks for 6 months and remained clinically well and the surface antigen was not detected at 13 months of age.

A more comprehensive randomized double-blind placebo controlled trial of hepatitis B immunoglobulin was carried out in Taiwan in an attempt to reduce the frequency of vertical transmission (Beasley and Stevens, 1978). The infants were given a single dose of one of three preparations, hepatitis B immunoglobulin, normal immunoglobulin without detectable hepatitis B surface antibody or heat-treated albumin. The most striking effect of hepatitis B immunoglobulin was a later onset of antigenaemia. There was also a slight but statistically insignificant lower rate of antigenaemia during the first year of life in the babies receiving hepatitis B immunoglobulin, and more of the babies in this group developed active antibodies at 10 months of age or older. However, infants who received hepatitis B immunoglobulin within the first 48 h of birth developed significantly fewer persistent infections. These results indicate that there is a need to determine the effect on vertical transmission of hepatitis B of a larger single dose of hepatitis B immunoglobulin administered at birth and of repeated doses.

Transfer factor

Immunotherapy with transfer factor has also been attempted. Transfer factor is a leucocyte extract entity that initiates and augments delayed type hypersensitivity (reviewed by Mazaheri *et al.*, 1977). Kohler *et al.* (1974b) treated a healthy chronic carrier of hepatitis B surface antigen with "immune" lymphocytes from an individual who had recovered from hepatitis B. There was a dramatic increase in the titre of the surface antigen and in transaminase levels within 24 h. It was suggested that the recipient's hepatocytes were transiently damaged by a cell-mediated immune response but that it was not sufficient to terminate the infection. Another patient, an infant who acquired hepatitis from her mother at birth, was given transfer factor prepared from the mother's leucocytes. The transfer factor caused on two occasions a prompt, moderate increase in both surface antigen and aminotransferase levels. Subsequently, liver function tests became normal and the titre of antigen was reduced by 95%. Jain *et al.* (1975) treated three patients with dialysates prepared from healthy donors and donors who had recovered from hepatitis B infection. One patient with hepatitis B surface antigen-positive chronic active hepatitis and cirrhosis did not respond to "normal" dialysable leucocyte extract, but injection of "specific" dialysable leucocyte extract resulted in a two-fold increase in T lymphocytes, as measured by sheep red blood cell rosetting. The second patient also did not respond to "normal" dialysable leucocyte extract, but showed a transient increase in aminotransferase activity. This suggested stimulation of cell-mediated immunity with a resulting hepatic damage. The third patient suffered from antigen-positive active chronic hepatitis complicated by primary liver cancer. This patient was being treated with corticosteroids and did not respond to either "normal" or "specific" dialysable leucocyte extract. There was no change in the serum titre of hepatitis B surface antigen in any of the patients.

There are no published reports on the prophylaxis or treatment of neonatal hepatitis B infection with transfer factor.

15

Hepatitis B virus and primary liver cancer

The geographical pathology of primary carcinoma of the liver presents intriguing epidemiological and demographic features. Primary liver cell carcinoma is uncommon in North America, Europe, the U.S.S.R. and Australia, and it appears to be relatively infrequent in Central and South America. In contrast, primary hepatocellular carcinoma is common in many communities in Africa and Southeast Asia and probably less so in parts of Japan. In some parts of Africa, liver cell carcinoma is the commonest type of cancer in adult males, and, in as many as 70–80% of these patients the tumour is found in livers with underlying macronodular cirrhosis. However, there is no evidence of fibrosis ·in the remaining patients and the high prevalence of liver cancer in some countries cannot be explained by a simple increase in the overall incidence of cirrhosis nor are the wide ranges in the geographical incidence of cirrhosis associated with similar variations in liver cancer. Nonetheless, it is widely considered that even if the relationship between cirrhosis and liver cell cancer may not be causal, the association may well represent different reactions to the same stimuli operating at perhaps different intensities or possibly to entirely different stimuli.

The role of viral hepatitis in the aetiology of chronic liver disease and primary hepatocellular carcinoma has been debated for many years (reviewed by Zuckerman and Dunne, 1974). The availability of specific laboratory tests for hepatitis B infection led to the demonstration of an excess prevalence of markers of active infection with this virus in patients with primary liver cancer when compared with the general population, with blood donors or with matched controls (World Health Organization, 1977). The more recent introduction of specific tests for hepatitis A virus

has not revealed progression of this infection to chronic liver damage. However, there is circumstantial evidence that a new form of viral hepatitis, which is unrelated to hepatitis A or hepatitis B, may lead to chronic liver disease. There is considerable evidence from many parts of the world, particularly from Africa, Asia, the Pacific and Mediterranean areas, of a marked excess of patients with ongoing or past infection with hepatitis B virus and primary liver cancer. Numerous studies have shown a highly significant excess of surface antigen, surface antibody and core antibody in these patients (see for example, Sherlock *et al.*, 1970; Reed *et al.*, 1973b; Nishioka *et al.*, 1975; Prince *et al.*, 1975b; Maupas *et al.*, 1975; Larouze *et al.*, 1976; Trichopoulos *et al.*, 1976; Okuda and Peters, 1976; Nazarewicz *et al.*, 1977; Tabor *et al.*, 1977c; Peters *et al.*, 1977; Szmuness, 1978; Zuckerman, 1978a).

These observations may be interpreted in several ways. Hepatitis B is ubiquitous in areas where macronodular cirrhosis and primary liver cancer are common. It is possible therefore that patients with hepatocellular carcinoma are unduly susceptible to hepatitis B infection and to the development of the persistent carrier state. Zuckerman (1974) posed the question of whether the infectious agent is the driver or the passenger. It has been suggested that an important factor in the possible aetiological association between hepatitis B infection and liver cell carcinoma may lie in an early age of exposure to infection (Zuckerman and Dunne, 1974). Indeed, in areas of the world where the prevalence of macronodular cirrhosis and primary liver cancer is high, infection with hepatitis B virus and the carrier state occur most frequently in early life, before the defence immune mechanisms have fully developed, and as many as 20% or more of the apparently healthy population may be carriers. It seems likely, therefore, that persistent hepatitis B virus infection occurs before the onset of chronic liver damage.

Another possibility is that persistent infection with hepatitis B virus leads to cirrhosis and that carcinoma then arises from regenerative nodules by mechanisms in which the virus is not involved. Such a mechanism may account for cases of liver cancer that occur in patients with alcoholic cirrhosis. However, this sequence does not explain liver cancer associated with persistent hepatitis B infection in about 20–30% of patients in the absence of cirrhosis.

Chronic hepatitis B infection can result in chronic liver damage and cirrhosis, but there is no information on the mechanism by which hepatitis B progresses to hepatocellular carcinoma. The kind of evidence

which is needed should show that the infection precedes the development of cancer, that the tumour cells contain apparent or cryptic virus-specific molecules or antigens, that the cancer cells produce the virus or viral specified antigens, and that the virus can transform cells in culture or induce hepatocellular carcinomas in experimental animals. Some progress is being made in this direction. Hepatitis B surface and core antigens have been found in normal or cirrhotic liver tissue in hepatocellular carcinoma, but there are only few unequivocal reports of finding these antigens in the actual tumour cells. Macnab et al. (1976), however, derived a cell line, designated PLC/PRF/5, from a patient with primary liver cancer whose blood had circulating hepatitis B surface antigen. The cells have been in culture for 30 months and resemble hepatocytes. Supernatant fluids from these cultures were found to contain the surface antigen and the titre of antigen increased with time. It was estimated that approximately 500 ng of antigenic material is produced per day per 10^6 cells over 20 passages. Immune electron microscopy revealed some clumping of pleomorphic spherical particles measuring 16–24 nm in diameter and the presence of some tubular forms (Stannard and Alexander, 1977).

However, virus-like particles were not found by thin-section electron microscopy in the cells despite the considerable production of particulate antigen by the cells, and only low antigenic activity was detected in disrupted cells. Saunders and Alexander (1977) used the technique of single cell isolation in soft agar to initiate three separate clones from the hepatoma cell line. All three clones produced hepatitis B surface antigen, and the amounts produced, either cumulatively or over short periods, did not differ significantly from the amounts found in the supernatant culture medium harvested from the parent cultures. Presumably the continued synthesis of the surface antigen is the result of abortive infection associated with the incorporation in the cell of some genetic code from the virus, since complete virus particles have not been detected in these cultures.

Hepatitis B virus contains a small circular double-stranded DNA and DNA polymerase. The endogenous DNA polymerase reaction uses the DNA as a template and this reaction can, therefore, be utilized as a specific probe by making radioactive virus DNA. Lutwick and Robinson (1977) studied the effect of virus DNA extracted from the liver tissue of patients with persistent hepatitis B infection by hybridization using the DNA product of the polymerase reaction. The amount of newly synthesized DNA was determined by the amount of nucleotide incorporation into an acid-insoluble form. DNA re-association kinetics were used to

determine the complexity of the new DNA. The results suggested that a unique region (or regions), corresponding approximately to 25–50% of the circular hepatitis B virus DNA template, was copied at one time during the reaction. DNA and RNA extracted from the livers of patients with persistent hepatitis B virus infection and chronic active hepatitis, and of particular interest hepatocellular carcinoma, accelerated significantly the rate of re-association of radioactive DNA from the virus particles. No enhancement was found when DNA extracted from uninfected healthy livers was used. The virus particle DNA base sequences were found to be alkali-stable. The presence of these sequences in alkali-stable, rapidly sedimenting DNA molecules suggests that some of the sequences are probably attached to host chromosomal DNA. This is consistent with the hepatitis B viral DNA being integrated into host DNA molecules as is the case with other DNA viruses (Sambrook et al., 1968). Other experiments indicated that the viral DNA base sequences can be detected in RNA extracted from liver infected with hepatitis B virus, but not in RNA from uninfected liver. These findings suggest the integration of hepatitis B virus DNA into high molecular weight cellular DNA and transcription into RNA in infected liver.

Of course, those who work with chemical carcinogens tend to believe that their favourite compounds, such as the polycyclic aromatic hydrocarbons, aromatic amines and amides, azo dyes, selenium or nitrosamines, are the most likely candidates for the principal environmental cause of cancer. Interest in the mycotoxins and the pyrrolizidine alkaloids continues. The aflatoxins, for example, have been shown to be the most potent hepatocarcinogens known for the rat and they also induce hepatic carcinoma in fish, birds and mammals, but so far not in primates. The extensive experimental data in animals form the basis for the suggested importance of fungal products in the aetiology of human liver cancer. It is also known that optimum conditions for the natural production of the aflatoxins and other mycotoxins are found in those areas of the world with a high incidence of liver cancer and furthermore such toxins have been isolated in some of these areas from human food. Yet, the quantity of aflatoxin to be ingested by an individual in the form of contaminated peanuts sufficient to cause necrosis of the liver of the type seen in experimental animals, assuming the dose to be similar, is likely to be in gross excess of that likely to be consumed, amounting to several kilogrammes per day.

Another compound which is a potent hepatocarcinogen is dimethylnitrosamine. The effect of hepatitis B virus as a co-carcinogen to dimethyl-

nitrosamine was investigated in rhesus and cynomolgus monkeys by Gyorkey *et al.* (1977). The group of monkeys given only hepatitis B showed no evidence of hepatocellular carcinoma over a period of 3 years. If the monkeys were injected with dimethylnitrosamine followed by the virus or the virus followed by nitrosamine, the virus did not act as a co-carcinogen nor did it shorten the incubation period of the carcinoma. If the animals were given nitrosamine shortly after birth they developed large confluent multifocal hepatocellular carcinoma which extensively replaced the liver parenchyma. Juvenile monkeys receiving nitrosamine first and then virus developed large, but well-circumscribed hepatocellular carcinoma. Juvenile animals inoculated with virus before nitrosamine developed postnecrotic cirrhosis, and multifocal hepatocellular carcinoma apparently developed in the cirrhotic nodules. Microscopically, the cirrhotic livers showed chronic hepatitis. It is possible, therefore, that the ingestion of low doses of such hepatocarcinogens in conjunction with other stimuli, over a long period of time, may be carcinogenic for humans.

In conclusion, although some progress is being made, the actual mechanisms involved in the pathogenesis of hepatocellular carcinoma remain unknown. It is possible that liver cancer is the cumulative result of several cofactors or hepatocarcinogens including infection with hepatitis B virus, genetic, immunological, nutritional and hormonal factors, mycotoxins and chemical carcinogens (Zuckerman, 1974). Outstanding perhaps among these factors are the geographical differences in the prevalence of hepatocellular carcinoma. These geographical differences may be environmental rather than ethnic or even genetic in nature, for while there is a similar low incidence of primary hepatocellular carcinoma in the negro and white populations of North America, the native African has a very much higher incidence. It also seems that persons born in China who migrated to Singapore are more prone to primary liver cancer than those born on the island itself. The evidence of a strong association between hepatitis B virus and primary liver cancer is compelling, and it appears that hepatitis B virus acts either as a carcinogen or as a co-carcinogen in persistently infected liver cells. The possibility should not be overlooked that hepatitis viruses other than type B hepatitis (and hepatitis A) may also play a part in the aetiology of chronic liver disease and as such may contribute directly or indirectly to the ultimate development of liver cancer. However, at present there are no specific serological or virological criteria for the identification of this additional type or types of human hepatitis viruses.

Laboratory tests for hepatitis A and the nature of the virus

Recent important advances in the laboratory studies of infection with hepatitis A virus include transmission to certain species of marmosets and susceptible chimpanzees and the identification of virus particles in faecal extracts. A number of serological tests for hepatitis A have been developed, including immune electron microscopy, immune adherence haem-agglutination, complement-fixation and radioimmunoassay. Enzyme linked immunosorbent techniques are currently under study.

IMMUNE ELECTRON MICROSCOPY

The discovery of the association between an antigen found in blood and hepatitis B stimulated numerous efforts to find a similar antigenic marker in hepatitis A, culminating in the finding of virus particles measuring 27 nm in diameter in faecal specimens obtained during the early acute phase of the illness in two out of four volunteers inoculated with the MS-1
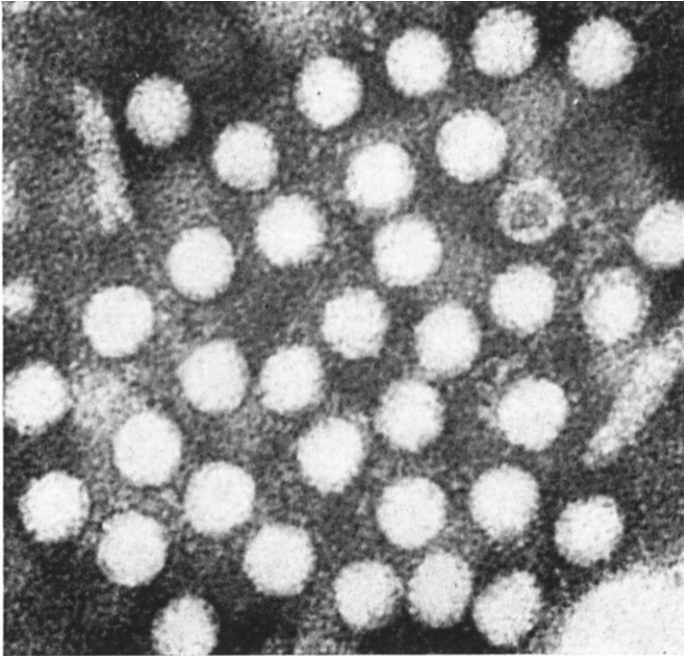
FIG. 16.1. A group of hepatitis A virus particles identified in faecal extracts prepared from chimpanzee George on day 26 of the incubation period. × 300 000. (From a series by A. Thornton and A. J. Zuckerman.)

strain of hepatitis A virus. The particles were aggregated, and the aggregates were composed of both "full" and "empty" particles heavily coated with antibody (Figs 16.1 and 16.2). These particles were not found in faecal specimens before infection. Faecal filtrates containing the 27-nm particles were used to examine by immune electron microscopy several groups of sera for the presence of antibody to this antigen. All six volunteers previously infected experimentally with hepatitis A developed serological evidence of infection, as judged by aggregation and antibody coating of the 27-nm particles. Similar results were obtained with sera from persons from two naturally occurring outbreaks of hepatitis A. None of the sera from the patients with hepatitis A contained detectable antibody to the 27-nm particles before exposure to the infection. There was no serological reaction between the hepatitis A faecal antigen and hepatitis B surface antigen and hepatitis B core antigen. Development of antibodies to the hepatitis A antigen particles has now been demonstrated in patients
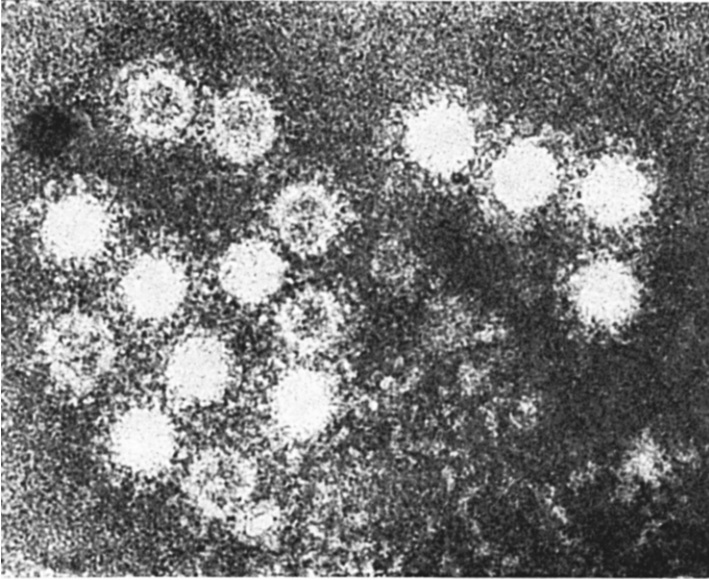
FIG. 16.2. Hepatitis A particles heavily coated with a "halo" of antibody. Both "full" and "empty" particles are present. There is a variation in particle diameter from 24 to 29 nm. × 300 000. (From a series by A. Thornton and A. J. Zuckerman.)

infected experimentally with the MS-1 strain, in patients from several different outbreaks of hepatitis type A infection and in the serum of random cases of hepatitis A. Such antibodies were not found in sera collected before the onset of illness. Further examination by immune electron microscopy of serial specimens of faeces from two volunteers who were infected experimentally in 1968 with the MS-1 strain of hepatitis A led to the detection of 27-nm virus-like particles in faecal extracts at least 5 days before elevation of the serum transaminases but not later than the day of peak transaminase levels. The sensitivity threshold of immune electron microscopy for detection of enterovirus was $10^{3.5}$ to 10^5 infectious viruses per ml and it was concluded therefore by analogy that the shedding of hepatitis A virus almost certainly begins earlier and lasts longer than detection of hepatitis A virus in the faeces by immune electron microscopy. The limitations of the cumbersome technique of immune electron microscopy for routine serological tests for antibody using the faecal antigen particles and indeed for searching for the antigen are obvious.

NON-HUMAN PRIMATES, HEPATITIS A AND THE DEVELOPMENT OF SEROLOGICAL TECHNIQUES

In the past the many attempts to transmit hepatitis A and B to various animal species, including non-human primates, failed or yielded inconsistent results. As regards hepatitis A, evidence of the successful transmission of human hepatitis to several species of marmosets was first provided in 1967, and the infection has been transmitted serially from animal to animal. Differences in susceptibility exist between the marmoset species, *Saguinus mystax* being the most susceptible. The MS-1 strain of hepatitis A was originally transmitted to marmosets by acute phase serum or plasma specimens from infected human volunteers, and the virus was neutralized *in vivo* in marmosets using convalescent sera but not pre-inoculation sera of the same volunteers. Subsequently, the CR 326 strain of hepatitis A was isolated from naturally occurring family outbreaks in Costa Rica. The CR 326 strain of hepatitis A was neutralized by convalescent sera from patients in the same outbreak, convalescent sera from volunteers infected with the MS-1 strain and pooled human immunoglobulin. Later, 27-nm virus particles were detected by electron microscopy in the sera and livers of *S. mystax* marmosets infected with the CR 326 strain.

Human hepatitis A virus has also been transmitted by several groups of investigators to chimpanzees shown to be free of the homologous antibody. Thornton *et al.* (1977) inoculated several chimpanzees found to be seronegative for hepatitis A by immune electron microscopy and by radioimmunoassay with known infective faecal extracts from several sources, including the MS-1 strain of hepatitis A virus, first passage material from chimpanzee George and a strain of hepatitis A virus obtained during a naturally occurring outbreak of infection in Germany. Elevated serum transaminase levels were found in the chimpanzees about 19 days after inoculation. Virus particles were found in faeces as early as 9 days after infection in one chimpanzee and by days 12 and 14 in the others. Excretion of virus continued for 9 to 19 days, and the maximum number of virus particles were found on the 17th to the 19th day after inoculation. Hepatitis A antibody was detected in the serum by immune electron microscopy and by radioimmunoassay during the period of incubation while virus particles were still being excreted in the faeces and coinciding approximately with the onset of biochemical evidence of liver damage (Fig. 16.1). A very close or identical morphological and sero-

logical relationship was demonstrated between hepatitis A virus isolated from humans and from experimentally infected chimpanzees.

Suitable species of non-human primates thus provided a model for experimental infection with hepatitis A and also a source of reagents for the development of specific serological tests for this virus.

COMPLEMENT-FIXATION

A specific complement-fixation test for human hepatitis A antibody was described by Provost et al. (1975) using as antigen liver extract of marmosets infected with the CR 326 strain of hepatitis A virus. The development of the complement-fixing antibody against hepatitis A correlated well with the development of neutralizing antibody. In most cases the highest titre of the complement-fixing antibody was attained within the first month after the onset of the acute illness and this antibody persists for at least several years. It is interesting to note that anticomplementary activity appeared to be present shortly after the onset of illness and it has been suggested that the high anticomplementary activity was due to the presence of circulating antigen–antibody complexes. This was further complicated by the frequent presence of antibody reacting with normal marmoset liver antigen, though at lower titre and without significant increase except when anticomplementary activity was present. Krugman et al. (1975) used the same source of antigen for complement-fixation and immune adherence haemagglutination for the measurement of hepatitis A antibody. The data obtained indicated that the immune adherence test was more specific, more sensitive and simpler to perform than the complement-fixation test.

IMMUNE ADHERENCE HAEMAGGLUTINATION

Miller et al. (1975) described a specific immune adherence assay for hepatitis A antibody. This method provides in general clearly defined endpoint titrations. Selection of the human red cells is most important since these cells vary in their sensitivity and indeed suitability for this assay. In addition there are occasionally problems with specificity and purification of the antigen from faeces (Moritsugu et al., 1976) and some preparations of the antigen do not work satisfactorily. Nevertheless, this is a most useful technique for the serological diagnosis of infection with hepatitis A virus. It was noted that antibody titres by immune adherence

haemagglutination correlated well with antibody ratings determined by immune electron microscopy.

IMMUNOFLUORESCENCE

Hepatitis A virus antigen was demonstrated by Mathiesen *et al.* (1977) by immunofluorescence using FITC-conjugated hyperimmune IgG and human convalescent IgG with high titre of hepatitis A antibody in liver biopsies from chimpanzees with experimental hepatitis A virus infection. The antigen was detected by immunofluorescence before the shedding of the virus in faeces, and before elevation of serum alanine aminotransferase or histological changes in the liver. The antigen was detected in the liver for 4 to 5 weeks and the last positive biopsy was obtained after alanine aminotransferase activity had returned to normal. In the early positive biopsies, the antigen was diffusely distributed in the cytoplasm of many cells in the liver, but later there was only focal distribution of the antigen in the cytoplasm of a few of the hepatocytes and Kupffer cells.

RADIOIMMUNOASSAY

Hollinger *et al.* (1975) described a microtitre two-stage immunoradiometric assay for hepatitis A antigen. The method involves the coupling of un-labelled antibody to an insoluble matrix. Antigen under test reacts with the antibody and the specific antigen is detected after interaction with a second radiolabelled hepatitis A antibody, with the uptake of radioactivity being proportional to the concentration of antigen taking part in the first antigen–antibody reaction. The effectiveness and specificity of this tech-nique for rapid and quantitative detection of hepatitis A virus antigen were demonstrated in specimens from infected marmoset liver, and faeces and serum from patients and chimpanzees. In addition, samples which were found negative by immune electron microscopy were found to contain significant levels of hepatitis A antigen by radioimmunoassay.

Purcell *et al.* (1976) used a blocking test in a microtitre solid-phase radioimmunoassay to measure hepatitis A antibody. Reduction in radio-activity of 40% or more compared with a negative serum is considered evidence for the presence of hepatitis A antibody. This technique appears to be the most sensitive method at present available for the assay of hepatitis A antigen and antibody.

Hall *et al.* (1977) compared the sensitivity of sandwich type solid-phase

radioimmunoassay for hepatitis A virus with immune electron microscopy. All samples found positive by immune electron microscopy were also positive by radioimmunoassay, and dilution experiments with positive faecal specimens showed that radioimmunoassay was at least as sensitive as immune electron microscopy. The pattern of shedding of hepatitis A virus during an outbreak of the infection in an institution for the mentally retarded varied among the patients. The virus was detected as early as 21 days before maximum elevation of serum alanine transferase and as late as 14 days after. In general, however, the maximum number of virus particles was found, by immune electron microscopy and by radio-immunoassay, during the incubation period 15 to 5 days before peak alanine transferase elevations.

Frosner et al. (1977) developed a solid-phase competitive type radio-immunoassay for the detection of hepatitis A antigen and antibody. This technique is based on a sandwich type assay using as the solid phase plastic beads coated with hepatitis A antibody, and radiolabelled IgG with high titre of anti-hepatitis A virus as the second antibody. The IgG fraction was prepared from a convalescent serum by ammonium sulphate precipitation followed by DEAE chromatography. IgG was labelled with ^{125}I by the method of Hunter and Greenwood. For the detection of hepatitis A antigen, faecal extracts are incubated with the antibody-coated beads. After washing with 0·9% normal saline, the beads are incubated overnight with ^{125}I-labelled antibody. Radioactivity bound to the beads is a measure of the amount of antigen present in the faecal specimen. For determination of hepatitis A antibody a measured quantity of suspension of positive faeces in 0·9% saline is used for the first incubation. The amount is chosen to give about 20 000 cpm with the radiolabelled IgG reagent after the second incubation. The inhibition of binding of ^{125}I-antibody to the plastic beads with the serum under test is a measure of the amount of hepatitis A antibody present in the specimen. Titres are expressed as 50% inhibition values.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA OR EIA)

Duermeyer et al. (1978) developed a sensitive, rapid and simple enzyme immunoassay for the detection of both hepatitis A virus and its antibody. Hepatitis A antigen is bound to the walls of polyvinyl chloride or poly-styrene microtitre plates coated with hepatitis A antibody. Hepatitis A

antibody conjugated with horseradish peroxidase is then allowed to react with the bound antigen. An enzyme substrate is used and the colour reaction can be read with the naked eye or with a spectrophotometer. The sensitivity of this technique is comparable with that of radio-immunoassay. Mathiesen *et al.* (1978) also found that the sensitivity of the enzyme immunoassay was comparable to that of solid-phase radio-immunoassay and immune electron microscopy for the detection of hepatitis A virus in faeces and hepatitis A antibody in serum. Because human faeces often react non-specifically in serological tests for hepatitis A virus, blocking of the reaction with hyperimmune hepatitis A antibody raised in a chimpanzee was used to confirm the specificity of the test. Locarnini *et al.* (1978) reported that enzyme immunoassay provided an accurate and rapid means of detecting hepatitis A virus in faeces. There was no appreciable loss in sensitivity by reducing the incubation time to 4 h (2 h for antigen binding and 2 h for blocking the reaction to establish specificity) so that samples could be tested the day after collection. It was also pointed out that this technique permits the rapid screening of large numbers of specimens with ease and if faecal specimens are available within 1 week from the onset of dark urine, detection of virus could have rapid diagnostic value. Furthermore, since the test is relatively cheap, reagents have a long shelf-life, sophisticated technical equipment is not required and the results can be read with the naked eye, making the test suitable for large-scale epidemiological studies. In this context, Crovari *et al.* (1978) noted that radioimmunoassay has been improved over the years, whereas enzyme immunoassay is a relatively recent innovation with considerable potential. In addition in some countries, including Italy, laws have been introduced which limit the use, handling and disposal of radioactive material so that enzyme immunoassay offers an attractive alternative test system.

DETECTION OF SPECIFIC HEPATITIS A IgM

The development of hepatitis A antibody early in the course of infection means that at least two samples drawn at appropriate intervals must be available for the diagnosis of recent infection and that serial dilutions will have to be tested. This can be extremely expensive (Berg *et al.*, 1979). A specific test for hepatitis A IgM, on the other hand, makes it possible to establish the diagnosis of recent infection on a single serum specimen. Several techniques are available for this purpose. The IgM-rich fraction

can be separated from serum on a sucrose density gradient and tested after treatment with 2-mercaptoethanol and without such treatment; but this is a laborious method. Duermeyer *et al.* (1979) described an enzyme immunoassay technique for the measurement of hepatitis A IgM, and solid-phase radioimmunoassay procedures are also under development. Hepatitis A IgM is detectable in serum for 45–60 days after the onset of symptoms.

THE NATURE OF HEPATITIS A VIRUS

Bradley *et al.* (1977) purified hepatitis A virus from the faeces of patients and from the faeces of experimentally infected chimpanzees and found that the virus had multiple buoyant densities in caesium chloride gradients. The largest proportion of virus was found most frequently at a buoyant density of 1·32–1·34 g cm^{-3}. However, the virus was also found frequently at higher densities of 1·36–1·37, 1·40–1·42 and 1·45–1·48 g cm^{-3}. It was considered that these findings were inconsistent with the view that hepatitis A virus is an enterovirus, since enteroviruses characteristically have a buoyant density of 1·34 g cm^{-3} in caesium chloride, whereas parvoviruses, on the other hand, possess multiple buoyant densities normally ranging from 1·35 to 1·47 g cm^{-3}.

Siegl and Frosner (1978) purified hepatitis A virus from faecal specimens obtained during an outbreak in Germany. The majority of the particles had a buoyant density in caesium chloride of 1·34 g cm^{-3} and a sedimentation coefficient in sucrose of about 160 S. In addition a distinct hepatitis A antigen was identified with a buoyant density of 1·305 g cm^{-3} and a sedimentation coefficient between 50 and 90 S. A further labile component accumulated in the density range between 1·38 and 1·44 g cm^{-3}. The diameter of the 160-S particles was 27–29 nm and was readily distinguished from that of the parvovirus marker (Lu III) used with a diameter range of 22–24 nm. The virus particles with the buoyant density of 1·34 g cm^{-3} released linear nucleic acid molecules in the presence of 4 M urea and about 90% formamide. The nucleic acid molecules had the kinked appearance characteristic of single-stranded RNA or single-stranded DNA and they were distinguished from the nucleic acid of phage λ added to the preparation as a marker for double-stranded configuration. Experiments in which hepatitis A particles were incubated at pH 12·9 for 30 min at 50°C showed that their nucleic acid molecules were hydrolysed as readily as the RNA genome of poliovirus

type 2 analysed in parallel. Both the single-stranded DNA of phage ϕX 174 and that of parvovirus Lu III, however, were not affected by this treatment, and the double-stranded DNA of phage λ was denatured to single-stranded molecules. It was concluded from these studies that hepatitis A virus contains a linear genome of single-stranded RNA and the virus should be classified with the picornaviruses.

Bradley et al. (1978) also concluded from the results of their studies that hepatitis A virus contains RNA. Hepatitis A virus particles with average diameter of 27–30 nm and with buoyant densities in caesium chloride of 1·34 and 1·45 g cm^{-3} purified from human faeces during common source outbreaks were shown to have identical sedimentation coefficients of about 157 S in neutral sucrose gradients. The heavy density particles (1·45 g cm^{-3}) sedimented at 157 S and 230 S in linear sucrose gradients, whereas the light density particles (1·34 g cm^{-3}) sedimented only at 157 S. Alkaline pH degradation of the light density particles showed 50% loss of the 157-S particles at pH 10·0 and complete loss at pH 11·0. Treatment of the heavy density particles revealed that a considerable proportion of the 157-S particles was not lost after degradation at pH 11·0. High density particles treated at pH 10·0 were sensitive to RNase digestion at concentrations as low as 10^{-4} μg ml^{-1}. DNase had no effect. Alkaline pH treatment of heavy density particles produced 157-S particles that were also sensitive to RNase.

Finally, Coulepis et al. (1978) purified hepatitis A virus from faeces obtained from three patients with naturally acquired infection. The purified virus preparations were subjected to discontinuous polyacrylamide gel electrophoresis in 0·1% sodium dodecyl sulphate. Three major polypeptides were separated with molecular weights of 34 000, 25 000 and 23 000. This polypeptide profile is specific for hepatitis A virus and the results closely resemble those reported for members of the *Enterovirus* genus within the Picornaviridae, although a fourth common low molecular weight polypeptide was not detected.

17 | Viral hepatitis in non-human primates

In the past, all attempts to transmit experimentally human hepatitis types A and B to many animal species, including non-human primates, failed or yielded inconsistent results. However, since 1961 more than 46 small clusters of cases of viral hepatitis have been reported in human beings in close contact with non-human primates. In most instances, the outbreaks occurred in association with recently captured juvenile chimpanzees (*Pan troglodytes*), but a gorilla (*Gorilla gorilla*), Celebes apes (*Macaca nigra*), gibbons (*Hylobates lar*), and woolly monkeys (*Brachyteles arachnoides*) have also been implicated. The more recent development of specific serological markers for hepatitis viruses A and B established the susceptibility of certain species of primates to these agents and revealed the prevalence of these antigens and antibodies among Old World and New World monkeys. Hepatitis B surface antigen or surface antibody and hepatitis A virus and antibody in captive chimpanzees are antigenically indistinguishable from the homologous antigens and antibodies obtained from human beings.

HEPATITIS A

The observations of non-human primate-associated hepatitis restimulated attempts to transmit human viral hepatitis to different species of monkeys (reviewed by Deinhardt, 1976). Working on the assumption that primates which had little or no contact with man were unlikely to have acquired immunity to the human hepatitis virus through subclinical infection, Deinhardt and his associates began to experiment with marmosets, a species of small South American monkeys. These animals have little or no contact with man in the wild and serological surveys of antibody confirmed that naturally acquired infection to common human viruses was rare. Biochemical and histological changes compatible with hepatitis were found in two species of marmosets, *Saguinus nigricollis* and *S. fuscicollis*, after the inoculation of acute phase serum or plasma from patients with viral hepatitis. There was no clinical evidence of infection in the marmosets, but liver damage was produced regularly in series five times, from marmoset to marmoset, using a pool of animal serum.

Further experimental studies demonstrated that marmosets inoculated with well-documented acute phase hepatitis A plasma from three infected human volunteers developed biochemical and histological evidence of hepatitis. Additional evidence was subsequently provided that marmosets of the species *S. mystax* were particularly susceptible to human hepatitis A virus. Biochemical evidence of liver damage and hepatic lesions were induced by the injection of serum from a pool of sera from volunteers with experimentally transmitted hepatitis A and by serum from pooled marmoset sera which had been passed five times in marmosets. Two marmosets which were previously inoculated with infectious marmoset serum did not develop evidence of hepatitis when challenged with human hepatitis A serum. In 1973, Holmes *et al.* provided the final piece of evidence on the susceptibility of certain species of marmosets to hepatitis A by neutralizing the infection in marmosets with convalescent human hepatitis A serum. The infectivity of acute phase MS-1 (hepatitis A) serum and its infectious fractions prepared by density gradient centrifugation in caesium chloride was neutralized with convalescent patient serum, whereas infectivity was unaffected by incubation with human serum albumin or by incubation with preinoculation serum from the same volunteers.

Some differences in susceptibility exist between species of marmosets, *S. mystax*, the white-moustached marmoset, being the most susceptible

and *S. (Oedipomidas) oedipus* the least susceptible. Since 1974 an embargo has been placed on the export of *S. mystax* from the Amazon basin. Provost *et al.* (1977) examined the rufiventer-like marmoset, designated as *S. labiatus, Marikina labiata* and *Jacchus rufiventer*, for its susceptibility to hepatitis A infection. The CR 326 strain of human hepatitis A virus, adapted to *S. mystax*, was passaged serially five times in rufiventer marmosets. The virus was readily adapted as shown by an increase in the proportion of infected animals, progressive shortening of the incubation period of the infection, and progressive increase in the proportion of liver extracts with hepatitis A virus.

Transmission of hepatitis A to chimpanzees
Human hepatitis A infection has also been transmitted to chimpanzees shown to be free of the homologous antibody (Maynard *et al.*, 1975; Thornton *et al.*, 1975; Dienstag *et al.*, 1975; Thornton *et al.*, 1977). Transmission experiments of different strains of hepatitis A virus were carried out by Thornton *et al.* (1977). Several chimpanzees found to be seronegative for hepatitis A antibody by immune electron microscopy and by radioimmunoassay were inoculated with known infective faecal extracts from several sources, including the MS-1 strain of hepatitis A virus, first passage from chimpanzee George (Thornton *et al.*, 1975) and a strain of hepatitis A virus (GBG) obtained during a naturally occurring outbreak of infection in Germany (Frosner *et al.*, 1977). Elevated serum transaminase levels were found in the chimpanzees about 28 days after inoculation. Hepatitis A virus particles were found in faeces as early as 9 days after infection in one chimpanzee and by days 12 and 14 in the others. Excretion of virus continued for 9 to 19 days, and the maximum numbers of virus particles were found on the 17th to the 19th day after inoculation. The buoyant density in caesium chloride of virus particles separated from faeces was $1 \cdot 31 – 1 \cdot 43$ g cm^{-3}. Most of the particles were found in the fraction with a density of $1 \cdot 34$ g cm^{-3}. Hepatitis A antibody was detected in the serum by immune electron microscopy and by radioimmunoassay during the period of incubation while virus particles were still being excreted in the faeces and coinciding approximately with the onset of biochemical evidence of liver damage. A very close or identical morphological and serological relationship was demonstrated between hepatitis A virus isolated from humans and from the experimentally infected chimpanzees. In a subsequent study, cyclic excretion of hepatitis A virus was demonstrated by Bradley *et al.* (1977). Two chimpanzees were

infected by the intravenous route with a strain of hepatitis A virus identified in Phoenix. Immune electron microscopy carried out on faeces collected daily from the chimpanzees revealed the presence of hepatitis A virus mainly on days 14 and 15 after inoculation. Examination by radioimmunoassay, however, showed the presence of hepatitis A in faeces with cyclic excretion of virus both earlier and later during the incubation period. Isopycnic banding in caesium chloride gradients revealed different periods of excretion of virus. "Empty" virus particles with a buoyant density of 1·30 g cm⁻³ were found in the pooled faecal extracts on days 9–11. Two viral antigen peaks, with buoyant densities of 1·29 and 1·33 g cm⁻³, were found in faecal extracts from days 14–15. Higher buoyant densities of 1·33 and 1·40 g cm⁻³ occurred with particles separated from faeces collected on days 17, 19 and 21 of the incubation period.

These various reports show that the chimpanzee can be a realistic source of hepatitis A virus for the development and application of serological tests. The nature of the illness in this species is so mild that the chimpanzees make a complete recovery. This point is of importance in conserving a species that may be endangered. However, chimpanzees susceptible to hepatitis A are difficult to find (A. Thornton, J. D. Almeida and A. J. Zuckerman, unpublished observations). At the same time, the detection of large numbers of virus particles only during the prodromal period of hepatitis A in the experimental model and also in natural infection of humans accounts for the difficulty in obtaining viral antigen from human sources for use as reagents for serological tests.

Transmission of hepatitis A to other non-human primates
Grabow and Prozesky (1975) reported that a small African non-human primate, with a wide geographical distribution, the lesser bushbaby (*Galago senegalensis*) may be susceptible to infection with hepatitis A virus. Confirmation of this report is awaited.

HEPATITIS B

There have been many unsuccessful attempts in the past to transmit hepatitis B virus to non-human primates. The finding of hepatitis B surface antigen and antibody in the serum of a small proportion of chimpanzees, orangutans and gibbons renewed interest in the possibility that such non-human primates might serve as an experimental model for

hepatitis B. Recent studies, employing sensitive assay methods for hepatitis B surface antigen and antibody, established the susceptibility of the chimpanzee to infection with the human hepatitis B virus. It would seem that much of the difficulty which had been experienced in the past was due to the unknown susceptibility of the animals before transmission studies.

Natural infection in non-human primates

Hepatitis B surface antigen and surface antibody have been detected in 6–12% of captive chimpanzees when tested by relatively insensitive techniques. Surface antibody has been found in a significant proportion of captive non-human primates when sensitive techniques such as passive haemagglutination and radioimmunoassay have been used. Antibody was found in the chimpanzee, orangutan, gibbon, baboon, Celebes ape, patas monkey, vervet, several species of macaque, mangabey, langur and in a number of species of New World monkey. Antibody was found in approximately 50% of chimpanzees examined but in less than 10% of most Old World and New World monkeys (World Health Organization, 1973), and the prevalence of antibody in both human and chimpanzee populations seems to increase with age.

Recently, an outbreak of hepatitis B was reported among the chimpanzees and other apes in the care of the Zoological Society of London (Zuckerman *et al.*, 1978). In February 1976, a serum sample was examined from a chimpanzee receiving treatment in the Animal Hospital for a chronic dermatosis of unknown aetiology. Electron microscopy revealed a large number of all three morphological forms of hepatitis B. The titre of the surface antigen in the serum determined by reverse passive haemagglutination was greater than 1 : 131 072, and DNA polymerase activity was elevated. Surface antigen was also detected repeatedly in the saliva, but not in urine or faeces. There was no past history of infection in this chimpanzee, which had been born at the Zoo and as far as was known she had not received human blood or blood products. Serological survey of the apes kept at the London Zoo revealed that surface antigen was present in very high titre (> 1 : 1 048 756 by reverse passive haemagglutination) in another four out of eight chimpanzees living as a group, and surface antibody was detected in the remaining four chimpanzees. The antigen was not found in the sera from two gorillas, 11 orangutans and two gibbons, although surface antibody was present in the serum of one gorilla and two orangutans.

Repeat samples from the chimpanzees confirmed these findings and established that four chimpanzees were persistent hepatitis B carriers. All three morphological forms of the antigen particles were found in their serum, the titres of the surface antigen were extremely high, ranging by reverse passive haemagglutination from $> 1 : 262\ 144$ to $> 1 : 1\ 048\ 756$, significant DNA polymerase activity was found in all four chimpanzees, and e antigen was detected in each case by immunodiffusion and rheophoresis. Surface antigen was also detected in the saliva of each chimpanzee, often to high titre, but the antigen was not found in the urine or faeces. The subtype of the surface antigen was $a_2 1 dw$. Hepatitis B surface antibody was never found by radioimmunoassay in any of the serial serum samples collected over a period of 2 years and immune complexes were not seen by electron microscopy. Hepatitis B core antibody was present in each case.

Serum transaminase levels (aspartic and alanine aminotransferase) were within the normal range for the species. The histological changes in liver biopsy specimens were consistent with a diagnosis of chronic persistent hepatitis in two chimpanzees and mild mesenchymal reaction with occasional inflammatory changes in the other two chimpanzees. Orcein staining revealed scattered liver cells with positive cytoplasmic staining for hepatitis B surface antigen.

The source of the outbreak at the London Zoo could not be traced. Carriers were not detected among the present staff. It should be noted, however, that hepatitis B surface antigen was found in two frozen serum samples from chimpanzees which had died in 1966 and 1972 and these, or indeed others, may have introduced the original infection. It is interesting that the subtype of antigen in all the cases was identical, i.e. $a_2 1 dw$.

An observation which is of particular interest is that three of the carrier chimpanzees born at the Zoo were the offspring of either a carrier mother or father. Furthermore, the implicated carrier mother and father were captured from the wild, the female in 1948 and the male sometime before 1964. Some years ago it was common practice for animal catchers and dealers to inoculate newly captured chimpanzees with pooled human blood for "protection against human disease" by passive transfer of antibodies. Since the blood for such inoculations was obtained in areas with high prevalence of endemic hepatitis B, it is possible that the chimpanzees were infected by this route. It is however, not yet known whethet hepatitis B infection may also be acquired in the wild. The second poinr

of interest is the possibility of perinatal transmission of hepatitis B virus in at least three of the chimpanzee matings, leading ultimately to the persistent carrier state.

Experimental transmission
The high frequency of naturally acquired infection with hepatitis B virus among the apes and the relatively mild nature of the illness in non-human primates were most probably responsible for the apparent failure of previous attempts to transmit this infection experimentally. However, since 1972 hepatitis B has been successfully induced in chimpanzees by several groups of investigators. In one comprehensive study six chimpanzees were selected for transmission studies of hepatitis B infection because they were seronegative for hepatitis B antigen and antibody using sensitive techniques. In the first experiment two chimpanzees were inoculated subcutaneously with an NIH plasma pool, which was shown many years ago to be highly infectious for humans (Murray *et al.*, 1955; Murray, 1955). In other experiments chimpanzees received plasma containing hepatitis B surface antigen collected 21 weeks after inoculation from one of the animals, and other animals were given material containing antigen from fractions obtained by rate-zonal centrifugation in sucrose and from an isopycnic banding in a sucrose gradient. Five of the six chimpanzees developed evidence of hepatitis B infection. In two of the five animals there was serological, biochemical and histological evidence of hepatitis. None of the chimpanzees had a raised concentration of serum bilirubin at any time, nor did they show any overt clinical manifestations of illness. The most reliable means of determining that infection had taken place seemed to be seroconversion, i.e. development of hepatitis B surface antibody.

Barker *et al.* (1975) inoculated intravenously 34 chimpanzees with sera containing hepatitis B surface antigen of subtypes *adw*, *ayw*, *adr* or *ayr*. Sera from the chimpanzees were examined before and after inoculation for surface antigen and surface and core antibody, and biochemical tests of liver function were carried out. In addition at least one liver biopsy was examined before inoculation. Twenty-nine of the chimpanzees became infected with hepatitis B virus, and in every instance the subtype of the surface antigen in the infected animal was the same as the subtype in the inoculum. Fourteen of the animals received the *ayw* MS-2 strain of hepatitis B virus. Evidence of infection was obtained in all animals inoculated with dilutions of up to 10^{-7}. However, there was no evidence

of infection in one animal that received the 10^{-8} dilution of this serum. There appeared to be a correlation between the dilution of the inoculum and the incubation period as measured by the interval before the appearance of detectable surface antigen and elevation of serum enzymes. Thus the average incubation period for antigen in animals inoculated with undiluted and 10^{-1} dilution of MS-2 serum was 4·8 and 10·8 weeks, and in those given 10^{-6} and 10^{-7} serum dilutions the respective incubation periods for transaminase elevations were 8·3 and 15 weeks. Similar correlations were observed in chimpanzees receiving dilutions of the *adw* and *adr* subtypes. The antigen persisted in the serum for 24 weeks and one chimpanzee became a carrier.

Again, the disease induced in chimpanzees was consistently mild, but it did encompass all of the typical biochemical and serological patterns of mild hepatitis B in humans. None of the animals became icteric nor demonstrated clinical evidence of overt disease such as lethargy, decreased food intake or weight loss. There appeared to be no correlation between the dose of virus and the severity of the disease as measured by the duration of antigenaemia or elevations of serum alanine aminotransferase.

Saliva and semen containing hepatitis B surface antigen and obtained from individuals clinically implicated in non-percutaneous transmission of hepatitis B were inoculated intravenously by Alter *et al.* (1977) into seronegative chimpanzees. Occult blood was not detected in the inocula. One chimpanzee was inoculated sequentially into separate veins of the chimpanzee with saliva from three individuals who developed acute hepatitis B. Surface antigen, *e* antigen, core antibody and surface antibody were detected in the chimpanzee and liver biopsies showed acute hepatitis which subsequently resolved. A second chimpanzee inoculated with semen developed the surface antigen and elevated serum alanine aminotransferase levels 4 weeks after inoculation and then died suddenly from an unidentified cause. Although infectivity of saliva and semen was demonstrated it is noted that it was not shown that saliva and semen are infectious by their natural routes, namely by oral and/or venereal contact.

A pool of whole-mouth saliva was collected by Bancroft *et al.* (1977) from five carriers of hepatitis B surface antigen, subtype *adr*. Occult blood was present in the saliva and immune electron microscopy revealed the morphological entities associated with hepatitis B including the complete virus. Two gibbons (*Hylobates lar*) were injected subcutaneously with 1·7 ml of the saliva pool every other day for 3 days. Twelve weeks after the first inoculation, one gibbon developed antigenaemia for 2 weeks

accompanied by elevated serum aminotransferase levels. Surface antibody was detected in all subsequent samples of serum. The second gibbon had elevated alanine aminotransferase and aspartate aminotransferase 10 weeks and 4 days after exposure. Surface antibody was detected after 22 weeks. In addition eight gibbons were exposed to the saliva pool by the oral and nasal routes by spray and by brushing of teeth or by ingestion of saliva injected into a banana, but none were successfully infected.

THE PATHOLOGY OF HEPATITIS A AND B IN CHIMPANZEES

Sequential liver biopsies from five chimpanzees infected experimentally with hepatitis B virus were examined by Hoofnagle et al. (1978) by immunofluorescence for surface and core antigens. Immunofluorescence was detected early in the course of infection, usually before transaminase elevation and before histopathological changes. By the time of peak enzyme elevation there was no detectable immunofluorescence for the surface and core antigens in the liver in three out of four chimpanzees with acute infection (the antigens were not detected in the liver of the fifth chimpanzee). Peak enzyme elevation and clinical disease did not correspond with the maximum reactivity of the surface and core antigens, but with the time of clearance or diminution of viral antigen expression in the liver.

Dienstag et al. (1976) compared the pathological changes in the liver after experimental infection of chimpanzees with hepatitis A and with hepatitis B. With type A hepatitis, the earliest abnormality was the detection of hepatitis A particles in the faeces 2 weeks after inoculation. The shedding of virus in the faeces reached a peak before significant elevation of alanine aminotransferase was noted 3 weeks after inoculation and persisting for about 1 week. Coincidentally hepatitis A antibody was detected by immune electron microscopy and morphological changes of acute hepatitis appeared. Hepatitis A antigen was not detected in the chimpanzee serum at any time during the investigation. The course of the serological, biochemical and morphological changes were qualitatively and quantitatively similar in the six chimpanzees infected.

With type B hepatitis the pathological changes varied considerably among the six chimpanzees, as is the case in humans. Typically, hepatitis B surface antigen was detected in the serum by radioimmunoassay 3 weeks after inoculation, preceding serum aspartate aminotransferase elevation

and morphological changes in the liver by 9 weeks. Antigenaemia persisted for 5 months. Hepatitis B core antigen was detected by immuno-fluorescence in liver cell nuclei, and surface antigen in the cytoplasm, for 2 weeks beginning a week before the histological changes in the liver. Core antibody appeared in the serum 3½ months after inoculation in the midst of surface antigenaemia. Surface antibody was not detectable until the seventh month after inoculation, 7 weeks after the disappearance of the surface antigen from the blood.

Basically, the morphological changes in hepatitis A and hepatitis B correlated well with biochemical evidence of liver injury. The pattern of changes in the liver were the same and consisted of conspicuous focal activation of sinusoidal lining cells; accumulations of lymphocytes and more histiocytes within the parenchyma, often replacing hepatocytes lost by cytolytic necrosis; mild diffuse hepatocytic changes with occasional coagulative necrosis in the form of acidophilic bodies; and focal regeneration and portal inflammatory reaction with alteration of bile ductules.

Hepatitis type A develops earlier and has a shorter duration of morpho-logical changes, while the lesions in hepatitis type B appear much later, linger on, fluctuate and regress slowly. There is a difference in distribution of lesions. In hepatitis A, the localization of parenchymal changes is predominantly periportal, whereas in hepatitis B the lesion is diffuse and, if anything, accentuated around the hepatic vein tributaries. Furthermore, in hepatitis B streaks of focal necrosis may extend from portal tracts to hepatic vein tributaries. The portal inflammatory reaction in hepatitis A is more severe than the parenchymal lesion.

Neither hepatitis A nor B in chimpanzees is as severe as hepatitis of any viral cause in humans. The main difference between hepatitis A and hepatitis B infection in chimpanzees and human hepatitis is the relative paucity of necrosis compared to the marked portal inflammatory reaction.

The ultrastructural changes in liver biopsies obtained from chimpanzees experimentally infected with hepatitis A and hepatitis B virus were described by Schaffner et al. (1977). Mitochondrial changes, which have not been noted in human beings, were seen at the time of maximum aminotransferase activity in the hepatocytes of chimpanzees infected with hepatitis A. Although the extent of involvement varied from cell to cell, all the cells were affected and had predominantly round rather than normally elongated mitochondria. The mitochondria were enlarged, the matrix was coarser than normal and not as uniformly homogeneous. The cristae of the mitochondria were thin, often curled and occasionally

circular. The rough endoplasmic reticulum appeared sparse. The smooth endoplasmic reticulum, mainly in the form of small round vacuoles rather than tubular forms, appeared to occupy more of the cytoplasm than normal, which enlarged the hepatocyte. Immediately before the maximum aminotransferase elevation, the smooth endoplasmic reticulum was clumped with tightly packed clusters of tiny vacuoles (or tubules?) in scattered cells. In contrast, minimal changes were noted in the cytoplasm of hepatocytes from chimpanzees infected with hepatitis B virus.

The nuclei of hepatocytes from chimpanzees infected with hepatitis A contained heterochromatic granules which measured 35–40 nm in diameter, were electron dense, appeared in small groups, were amorphous in shape and some had an outer electron-dense shell. These structures were not detected in liver cell nuclei of chimpanzees infected with hepatitis B, but the characteristic 27-nm core antigen particles were found.

TRANSMISSION OF HEPATITIS B TO RHESUS MONKEYS

The successful serial transmission through five passages of hepatitis B virus in the rhesus monkey (*Macaca mulatta*) was reported by London *et al.* (1972). Hepatitis B surface antigen, or antibody, or both, were detected at each passage level. The antigen was demonstrated transiently by radioimmunoassay only. Antibody was detected by passive haemagglutination and by radioimmunoassay. The infection in the rhesus monkey was not apparent and it was not associated with biochemical evidence of liver disease nor with obvious histological changes in the liver. However, the period of incubation, measured by the production of surface antigen and the antibody responses recorded, was similar in pattern to that observed in humans following either natural or experimental infection with hepatitis B virus.

Zuckerman (1972) postulated that the high prevalence of hepatitis B surface antigen in many tropical countries may be related to changes in the immunological response associated with a background of repeated parasitic infection and in particular to the immunosuppressive effect of malaria. An extension of this hypothesis was the induction of chronic infection with *Plasmodium inuei* in the rhesus monkey in an attempt to enhance the susceptibility of this species of non-human primate to hepatitis B virus (Zuckerman *et al.*, 1975; Scalise *et al.*, 1978).

Sera from young rhesus monkeys were screened for hepatitis B surface

antigen and hepatitis B surface antibody by solid-phase radioimmunoassay before they were admitted to the study. The animals were divided into small groups consisting of uninoculated controls, those infected chronically with *Plasmodium inuei*, a group inoculated intravenously with 0·25 ml of a well characterized serum known to have induced recently clinical post-transfusion hepatitis in humans and containing hepatitis B surface antigen subtype *adw*, and a small number of animals inoculated with the same hepatitis B infective serum after chronic infection with malaria was fully established. Hepatitis B surface antigen was detected in the serum of four out of six rhesus monkeys previously infected with malaria. The antigen was demonstrated in one animal on the day of inoculation, presumably due to the antigen present in the original inoculum and the antigen was subsequently found repeatedly in the serum of this subject for 45 days. Surface antibody was first detected 12 days after inoculation and it has persisted in the serum so far for 18 months. In another monkey the antigen was first detected 18 days after inoculation and it persisted for 30 days. An antibody response followed 54 days after inoculation. In the third animal the antigen was first found 4 days after inoculation and it remained in the circulation for 45 days without a detectable antibody response. Antigen was detected for 1 day only 25 days after inoculation in another animal, again without an antibody response. There was no detectable antigen or antibody in the remaining two monkeys of this group. Hepatitis B surface antigen was not found by radioimmunoassay in any of four animals inoculated with infective hepatitis B serum only. An antibody response was elicited in only one of these rhesus monkeys, 54 days after infection, and the antibody has persisted for some months.

Serum containing hepatitis B antigen was collected from two of the successfully infected rhesus monkeys and 1 ml was administered intravenously to three animals previously infected chronically with *Plasmodium inuei*. Hepatitis B surface antigen was detected in the serum of one of these animals 49 days after inoculation, but an antibody response has not been observed.

Infection with hepatitis B in the rhesus monkeys was subclinical, specific histological changes in the liver were not seen and the surface antigen was not detected in the liver by staining with orcein or with aldehyde fuschin.

Leucocyte macrophage migration tests were carried out at various intervals after inoculation with hepatitis B serum on peripheral blood leucocytes obtained from three rhesus monkeys with an established chronic

infection from *Plasmodium inuei*. Marked inhibition of cell migration was found in one monkey 2 months after inoculation with hepatitis B and the inhibition persisted for at least 7 months. Inhibition of leucocyte macrophage migration was detected in the other two animals 7 months after inoculation. Hepatitis B surface antibody was not detected by radioimmunoassay in any of these three monkeys. Lymphocyte transformation following stimulation by phytohaemagglutinin was normal in each case suggesting that the T cell function was unimpaired. Two monkeys inoculated with hepatitis B serum but in the absence of malaria served as controls. There was no inhibition of leucocyte macrophage migration at any time and lymphocyte transformation was normal throughout the period of observation.

The effect of malaria on humoral immune responses to different antigens is known to vary widely. Greenwood (1974) discussed the evidence obtained in experimental animals and in humans suggesting that malaria exerts its immunosuppressive effect by acting either on the B lymphocytes, on macrophages or on cell co-operation. There is good experimental evidence that although malaria enhances phagocytosis by macrophages it has a deleterious effect in other, more specialized, macrophage functions. Greenwood considered, therefore, that defects in the handling and processing of antigen by macrophages may play an important part in producing the immunosuppressive action of malaria. Further studies are clearly required to elucidate the mechanism of enhancement of susceptibility to infection with hepatitis B virus in chronic malaria. However, the rhesus monkey model is not sufficiently sensitive for studies of hepatitis B virus.

THE THIRD FORM OF HEPATITIS (NON-A : NON-B HEPATITIS) IN CHIMPANZEES

The diagnosis of a third form of hepatitis, non-A : non-B hepatitis, has depended on the exclusion of infection with hepatitis A and hepatitis B by means of sensitive laboratory tests. Non-A : non-B hepatitis was recently transmitted to chimpanzees by several groups of investigators in the United States and in Britain.

Alter *et al.* (1978) induced biochemical and histological hepatitis in five chimpanzees, using plasma or serum from four patients with acute or chronic non-A : non-B post-transfusion hepatitis and from a blood donor implicated in two cases of post-transfusion hepatitis. The histological

lesions in the chimpanzees were identical to those found in hepatitis B, involving the entire hepatic lobule and with streaks of focal necrosis extending into the central zone. The histological changes ranged from mild to conspicuous hepatitis and generally correlated with the degree of alanine aminotransferase elevation. Tabor *et al.* (1978) used serum from a patient with chronic hepatitis, whose blood transmitted acute hepatitis to a nurse following accidental inoculation with a needle, and serum from two former blood donors who transmitted clinical hepatitis to recipients, to induce biochemical and histological hepatitis in chimpanzees. None of the chimpanzees developed serological evidence of infection with hepatitis A or hepatitis B viruses nor with cytomegalovirus or EB virus.

Data obtained by the Transfusion-transmitted Viruses Study Group in the U.S.A. revealed that approximately 85% of their cases of post-transfusion hepatitis were unrelated to hepatitis virus A or B. The incidence of these non-A : non-B hepatitis infections in recipients of blood at the four participating centres ranged from 5·4–18·5%, with a mean rate of 12·6%. Sera from three blood donors who were involved in two cases of non-A : non-B hepatitis were purposely selected by Hollinger *et al.* (1978) because their alanine aminotransferase levels were normal and they induced hepatitis in chimpanzees, indicating that apparently healthy carriers exist among blood donors. In addition, the development of liver disease in two chimpanzees after inoculation of sera collected from blood recipients at least 12 days before clinical illness provided evidence that an early period of viraemia exists in this infection. Infection was also induced by serum with elevated alanine aminotransferase levels.
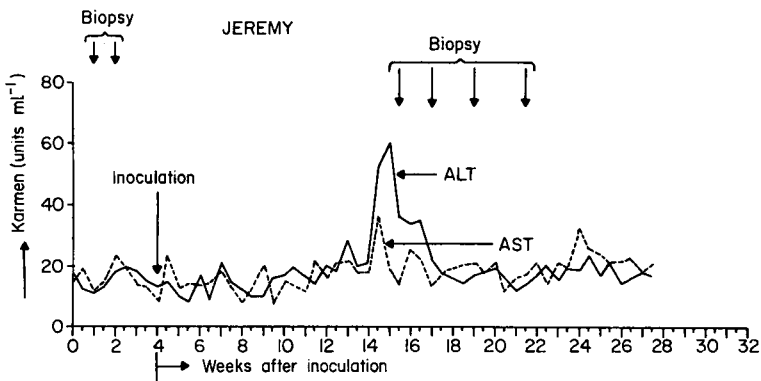
FIG. 17.1. Serum aminotransferases in chimpanzee Jeremy inoculated with Factor IX implicated in several severe cases of acute non-A : non-B hepatitis.
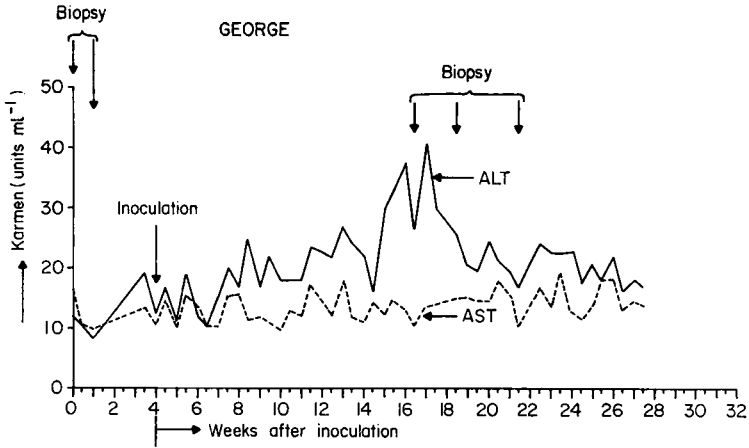
FIG. 17.2. Serum aminotransferases in chimpanzee George following inoculation with another commercially prepared batch of Factor IX upon which there were no reported adverse reactions. Acute hepatitis, confirmed histologically, developed after 10 weeks incubation.
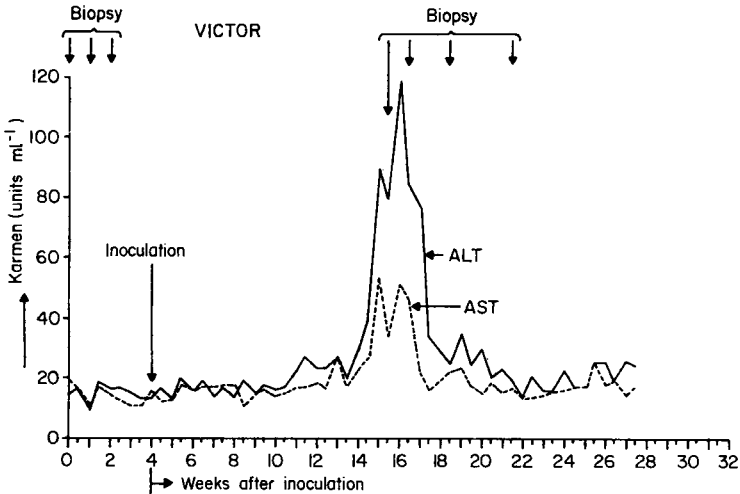
FIG. 17.3. Chimpanzee Victor was inoculated intravenously with 2 ml of documented infective non-A : non-B plasma.

A commercially prepared human blood product consisting of pro-thrombin (Factor II), Christmas factor (Factor IX) and Stuart–Prower factor (Factor X), that had been implicated in several severe cases of acute non-A : non-B hepatitis in patients in London, was inoculated by

Tsiquaye et al. (1978b) into a chimpanzee (Jeremy) held at the Primate Unit of the London School of Hygiene and Tropical Medicine. Another chimpanzee (George) received an identical product which had not been implicated in clinical cases of human hepatitis. The material in each case was given intravenously in a dose of 1500 units dissolved in 30 ml of water for injection. The positive control inoculum consisted of 2 ml of documented infectious non-A : non-B plasma (kindly provided by Dr H. Alter and Dr R. Purcell). This plasma was administered intravenously to chimpanzee Victor. The clinical charts of each chimpanzee are shown (Figs 17.1, 17.2 and 17.3). The following features were noted:

1. The incubation period was consistent at about 10 weeks.
2. Alanine aminotransferase (ALT) levels were higher than those of aspartate aminotransferase (AST), and are a useful biochemical marker for this form of hepatitis.
3. Preinoculation biopsies revealed normal liver histology, but at the height of alanine aminotransferase elevations the histological appearances were consistent with acute viral hepatitis, including collections of mainly mononuclear inflammatory cells in the portal tracts, inflammatory infiltrates and focal intralobular areas of necrosis. Kupffer cells were very prominent.
4. Chimpanzee George had already suffered from hepatitis A and hepatitis B, and Jeremy and Victor had preinoculation hepatitis A antibody. There were no changes in the markers of hepatitis A and B infection throughout the course of these transmission experiments.
5. An apparently normal batch of this blood product caused mild hepatitis in chimpanzee George, raising doubts concerning the safety of such plasma fractions.

Bradley et al. (1979) transmitted non-A : non-B hepatitis to four chimpanzees by infusion of three batches of antihaemophilic factor (Factor VIII) implicated in hepatitis in two human recipients. Acute phase plasma from one of the infected chimpanzees induced this new form of hepatitis in two other chimpanzees about 3 weeks after inoculation. In addition, examination by immune electron microscopy of an open liver wedge biopsy obtained during the acute illness from one chimpanzee revealed virus particles measuring 25–30 nm in diameter. Two other chimpanzees inoculated with a caesium chloride gradient fraction, with an average buoyant density of $1 \cdot 31$ g cm^{-3} and containing these virus particles, developed biochemical and histological evidence of non-A : non-B hepatitis after a shorter incubation period of about 15 days.
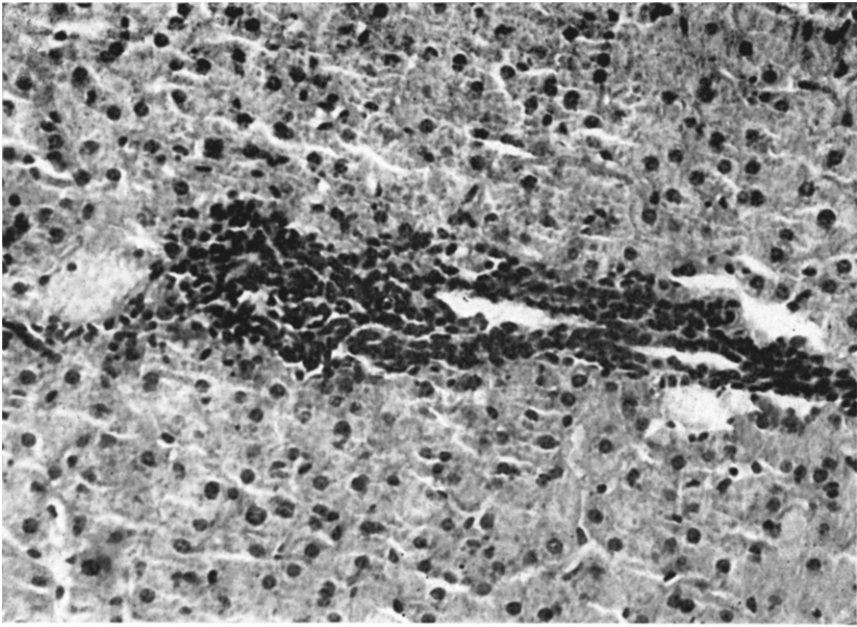
FIG. 17.4. The histological appearance of a liver biopsy from a chimpanzee during peak elevation of aminotransferase, showing inflammatory cell infiltrate of the portal tract and prominence of Kupffer cells. Haematoxylin and eosin × 263.
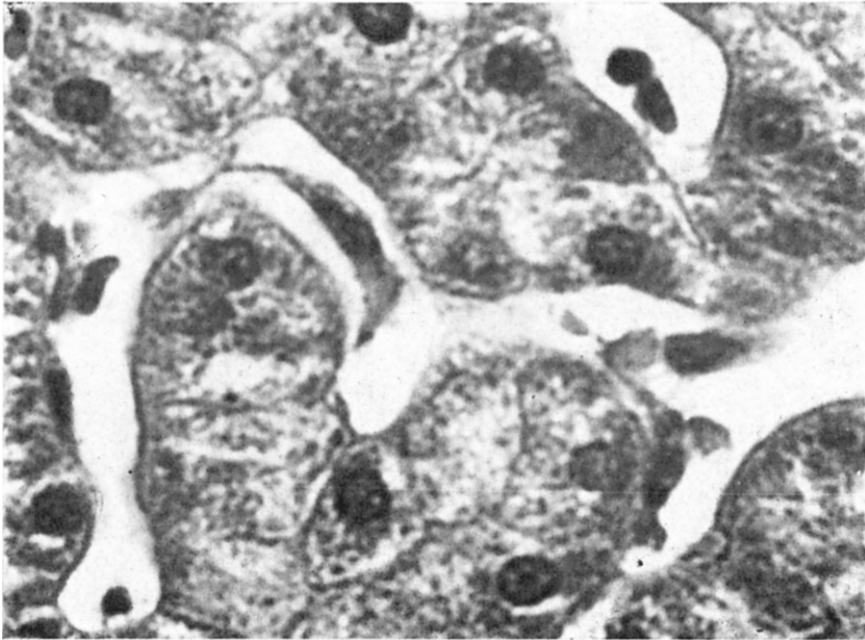
FIG. 17.5. The prominence of the Kupffer cells during the course of acute hepatitis in the chimpanzees with non-A : non-B hepatitis is well illustrated in this section. Haematoxylin and eosin × 525.

These studies show that chimpanzees are susceptible to the new form of human viral hepatitis. Challenge experiments should indicate whether one or more viruses are responsible for the so-called non-A : non-B hepatitis.

18

Non-A : non-B hepatitis

The identification of specific viral antigenic markers of hepatitis A and hepatitis B in recent years has permitted the development of sensitive laboratory tests. These in turn have led to a better understanding of the epidemiology, pathogenesis, immunology and nature of these infections. In addition, the specific diagnosis of hepatitis type A and hepatitis type B has revealed a previously unrecognized form of hepatitis which is clearly unrelated to either type. It is now the most common form of hepatitis occurring after blood transfusion in some areas of the world (WHO Expert Committee on Viral Hepatitis, 1977). Although there are as yet no specific laboratory tests for identifying this new form of hepatitis, recent studies nevertheless stress the importance of this infection. Results obtained from several recent surveys of post-transfusion hepatitis in the U.S.A. provide strong epidemiological evidence of "guilt by association" of an infection of the liver referred to as non-A : non-B hepatitis.

NON-A : NON-B POST-TRANSFUSION HEPATITIS

Prince *et al.* (1974) reported that an agent other than hepatitis B appeared to be the cause of 36 (71%) of 51 cases of post-transfusion hepatitis identified during a prospective serological follow-up of 204 patients who underwent cardiovascular surgery. The epidemiological and clinical features of the infection were inconsistent with hepatitis A and the hepatitis was not associated with cytomegalovirus. It was suggested that a

large proportion of long-incubation post-transfusion hepatitis is unrelated to hepatitis B and that it was caused by a hepatitis virus(es) type C. Feinstone *et al.* (1975) investigated 22 patients who developed transfusion-associated hepatitis after cardiac surgery for evidence of infection with hepatitis A and hepatitis B viruses, cytomegalovirus and EB virus. Hepatitis B surface antigen and surface antibody were not detected by radioimmunoassay, although antigen-positive hepatitis occurred as a second hepatitis infection in one patient. No evidence of infection with cytomegalovirus or EB virus was obtained. Paired sera from each patient were tested by electron microscopy against hepatitis A antigen-containing faecal filtrate for evidence of antibody conversion. Thirteen patients had hepatitis A antibody before the transfusion and a rise in titre was not demonstrated. Hepatitis A antibody was not detected in the pre-transfusion sample of the nine remaining patients and none of these showed seroconversion. This may be taken as evidence that the episode of post-transfusion hepatitis was not caused by hepatitis A virus. The incubation periods of these 22 episodes of hepatitis ranged from 2 to 15 weeks. Twelve of the episodes had incubation periods of 7 weeks or longer. The 22 patients investigated received a total of 395 units of blood, none of which induced a serological response to hepatitis A in the recipients. It was considered likely that at least a proportion of such cases of transfusion-associated hepatitis, unrelated to the antigens of hepatitis A and B, is caused by other unidentified infectious agents.

Dienstag *et al.* (1977) examined for hepatitis A antibody sera from 32 patients with transfusion-associated hepatitis, all of whom received multiple transfusions of blood pre-tested by radioimmunoassy for hepatitis B surface antigen. It was previously shown by serological tests that none of these patients had evidence of infection with hepatitis B, cytomegalovirus or EB virus. The incubation periods ranged from 2 to 15 weeks between transfusion and onset of hepatitis. Pre-transfusion and late convalescent pairs of sera were tested by immune adherence haemagglutination, using hepatitis A antigen purified from faeces. Twelve patients did not have detectable hepatitis A antibody in either the pre-transfusion or the convalescent samples. The other 20 patients had hepatitis A antibody in the pre-transfusion sample; in 18 patients there was no significant change in antibody titre, but two patients had an antibody titre of 1 : 100 before transfusion and 1 : 400 5–6 months later. However, these rises in antibody titre are feeble and a typical response to hepatitis A infection in which increases of a 1000-fold or

greater are normal is the rule. This study therefore also excluded hepatitis A virus as the cause of post-transfusion hepatitis in these cases. It was noted that among patients in the U.S.A. who developed hepatitis after the transfusion of blood prescreened by radioimmunoassay for hepatitis B surface antigen, 80–90% could not have been due to infection with the hepatitis A or hepatitis B viruses.

In the series of volunteer studies carried out in the U.S.A. in the early 1950s, serum samples from six blood donors, implicated in the transmission of post-transfusion hepatitis after a single unit transfusion, were each inoculated into groups of 10–20 male prisoners. Sera from five of the implicated donors induced hepatitis in recipients. The serum samples collected during these studies were tested by Hoofnagle et al. (1977) for evidence of infection with hepatitis virus types A and B, cytomegalovirus and EB virus. Two of the donor sera contained markers of hepatitis B virus and transmitted hepatitis B infection to all susceptible recipients, two of whom developed hepatic coma. The sera from the remaining three donors did not contain hepatitis B markers, but these sera were nevertheless infectious. A form of hepatitis was transmitted to nine volunteer recipients which could not be attributed to hepatitis A, hepatitis B nor to infection with cytomegalovirus or EB virus. The incubation period ranged from 18 to 89 days and the clinical illness was similar in most respects to hepatitis B. Of added significance was the finding of some evidence of persistent liver damage in one out of ten recipients who developed hepatitis type B and in two out of six volunteers who developed the unspecified type of hepatitis (non-A : non-B hepatitis).

In a survey on the efficacy of immunoglobulin for the prevention of post-transfusion hepatitis, Knodell et al. (1977) identified 44 patients with acute non-A : non-B hepatitis. Sixteen of the patients had persistent biochemical evidence of liver damage 6 months after the acute illness. In ten patients the abnormality of liver enzymes continued for 1–3 years and a liver biopsy in each patient revealed histological evidence of chronic liver damage. It was further observed that nine out of 22 patients who developed evidence of chronic hepatitis received albumin solution as a placebo, whereas only one of the 11 patients who were given normal immunoglobulin developed chronic hepatitis and none of 11 patients who received hepatitis B immunoglobulin developed any signs of the illness.

A nosocomial outbreak of parenterally transmitted hepatitis involving nine patients with bone-marrow transplants, 26 normal blood platelet donors and one other person occurred in 1972 in an oncology unit. At the

time there was no laboratory evidence to implicate infection with hepatitis B virus, cytomegalovirus or Q fever. The available sera were retested by Meyers *et al.* (1977) for hepatitis A antibody and antibody to EB virus from eight transplant patients who had clinical hepatitis, eight normal donors, two of whom had clinical hepatitis and the remaining six sub-clinical infection, and one donor with no clinical or biochemical evidence of infection. The incubation period of the patients now described was 22–37 days with a mean of 27 days. Secondary spread of infection was prevented by normal human immunoglobulin. Ten of these patients who developed biochemical or clinical evidence of hepatitis, or both, had no antibody response to hepatitis A virus as measured by immune adherence haemagglutination, and five other patients had pre-existing hepatitis A antibody and, therefore, are presumed to have been immune. There was no evidence of infection with EB virus or cytomegalovirus. This outbreak can, therefore, be ascribed to non-A : non-B hepatitis.

A more recent outbreak of non-A : non-B post-transfusion hepatitis was reported from a small hospital in Florida (Weekly Epidemiological Record, 1979). Eleven cases of hepatitis were identified and all of the patients received multiple blood transfusions within 3 months of the onset of symptoms. Eight of these 11 cases were caused by hepatitis agents other than hepatitis B, hepatitis A, cytomegalovirus and EB virus. All of the eight patients with non-A : non-B hepatitis were jaundiced, and in this outbreak the risk of post-transfusion hepatitis was associated with the transfusion of blood from commercial donors.

SPORADIC FORM OF NON-A : NON-B HEPATITIS

Further evidence for the existence of a third type of human viral hepatitis was provided by Villarejos *et al.* (1975). In the course of epidemiological investigation of hepatitis in Costa Rica, sera from 103 patients were examined for hepatitis A antibody by immune adherence haemagglutination, for hepatitis B surface antigen and antibody by radioimmunoassay and passive haemagglutination respectively, for antibodies against cytomegalovirus by complement-fixation, and for infectious mononucleosis by the Hoff and Bauer slide test. Infection with these viruses was thus excluded in 11 patients with hepatitis. In one other patient antibody against cytomegalovirus was not present in the first specimen tested but the antibody appeared during convalescence so that this patient might have contracted cytomegalovirus hepatitis or a concurrent infection. These

patients had not received blood transfusions and there was no indication of exposure to hepatotoxic agents, including alcohol and narcotic drugs. The available evidence indicated person-to-person transmission, and the illnesses were as often subclinical as clinical. All the patients had been previously diagnosed on epidemiological, clinical and biochemical grounds as suffering probably from hepatitis A; however, the illness in these patients was evidently neither hepatitis A nor hepatitis B.

In another study on the seroepidemiology of viral hepatitis in Costa Rica, Villarejos et al. (1976) described five patients with hepatitis who did not have any evidence of infection with hepatitis type A or type B. Indeed, three had pre-existing hepatitis A antibody and another had pre-existing hepatitis B surface antibody. None developed detectable hepatitis B antigenaemia and none had an increase in antibody titres against hepatitis A or B during convalescence. Four of these five patients had subclinical infections and the fifth had anicteric hepatitis.

Mosley et al. (1977) investigated for serological evidence of infection with hepatitis A and hepatitis B 13 patients who were re-admitted to a hospital in Los Angeles with repeated attacks of acute hepatitis. These patients had a total of 30 episodes of acute viral hepatitis. Immune electron microscopy was used for testing for hepatitis A antibody, radio-immunoassay for hepatitis B surface antigen and core antibody and passive haemagglutination for surface antibody. Serological tests were also carried out for infectious mononucleosis and cytomegalovirus. Twelve of the 13 patients had evidence that hepatitis B virus was the cause of one of the multiple episodes of acute hepatitis and only two patients had evidence that one of their infections was hepatitis A. One possible explanation for multiple attacks of acute hepatitis would be clinical recurrences of chronic infection. This appears unlikely in view of the following observations:

1. Each episode was a distinct departure from good health, and was accompanied by typical prodromes of acute viral hepatitis.
2. All liver biopsies in subsequent episodes had the features of acute hepatitis, without evidence of chronicity.
3. There were no statistically significant changes in serological indecises of hepatitis A or B during "non-A : non-B" episodes.
4. The serologically identifiable episodes did not necessarily precede the unidentified bouts.
5. Multiple episodes are most commonly observed in drug addicts using the parenteral route, which is to be expected from the range and intensity of their exposure.

These observations suggest that in addition to a third human hepatitis virus, there may well be a fourth serologically distinct hepatitis agent. Dienstag *et al.* (1977) investigated the cause of acute hepatitis in 45 patients admitted to a hospital in Los Angeles in whom the diagnosis of hepatitis B was excluded by tests for hepatitis B surface antigen, surface antibody and core antibody. However, subsequent tests revealed a rise in hepatitis B core antibody in two patients followed by the appearance of surface antibody, and an unchanging core antibody titre in another three patients placed the latter in a group which could not be classified. Hepatitis A was diagnosed in 20 (44%) of the 45 patients on the basis of an increase in titre of antibody to hepatitis A virus by immune adherence haemagglutination. There was no laboratory evidence of infection with hepatitis A, hepatitis B, infectious mononucleosis or cytomegalovirus in the remaining 20 patients who were classified as having non-A : non-B hepatitis. Clinically, non-A : non-B hepatitis could not be distinguished from hepatitis type A. However, in the patients with hepatitis A there was a preponderance of men with a high incidence among young adults of both sexes. Among those with non-A : non-B infection, the men, who were in the minority, were also young. The predominance of women among this group was striking and most of the women were over 35 years old. None of the hepatitis A cases, but eight of the 22 cases of non-A : non-B hepatitis, followed exposures typically associated with hepatitis B, namely blood transfusion, plasmapheresis donations and occupational contact with sick or convalescent patients.

These reports suggest the following: firstly, a carrier state of this newly recognized hepatitis agent must exist since the infection is transmissible by blood transfusion and probably by other routes from apparently healthy persons; secondly, the infection must be common since pooled immunoglobulin appears to contain antibody as shown by the prophylactic efficacy of immunoglobulin in one study and indeed a sporadic form of this infection has been reported from several countries; and thirdly, it seems that this infection may progress to chronic liver disease.

BLOOD FRACTIONS AND NON-A : NON-B HEPATITIS

Cryoprecipitate for the treatment of haemophilia is prepared from one or two donations of blood and carries therefore a relatively low risk of hepatitis. Factor VIII, on the other hand, has considerable advantages but because commercial concentrates are prepared from large pools of plasma

of up to 6000 l obtained by plasmapheresis from paid donors, the risk of transfusion hepatitis is much higher. Craske *et al.* (1975), for example, described an outbreak of both short-incubation non-B hepatitis and hepatitis B in southern England associated with the use of three out of four batches of a commercial Factor VIII concentrate. Seven cases of non-B hepatitis and four of hepatitis B occurred within 6 months of the first use of this product. Two patients contracted both types of hepatitis, so that nine patients out of a total of 20 patients regularly attending the haemophilia centre became ill. Eighteen of the 20 patients received the implicated product. Thus the overall attack rate in the Bournemouth outbreak was 50% for all types of hepatitis.

The high risk of transmitting viral hepatitis was identified shortly after the introduction of treatment with plasma components prepared from pooled human plasma (see Chapter 3). The report of Hoofnagle *et al.* (1976) is one example. Commercially prepared batches of plasma derivatives fractionated in the U.S.A. between 1957 and 1975 were tested for hepatitis B surface antigen by radioimmunoassay. The antigen was detected in 970 (69%) of 1402 batches of plasma protein fraction, in 81 (40%) of 199 batches of Factor IX concentrate, in 414 (20%) of 2064 batches of normal albumin, in 203 (13%) of 1550 batches of antihaemophilic factor (Factor VIII), and in 15 (3%) of 529 batches of immunoglobulin prepared by Cohn fractionation. A sharp decrease in the prevalence of surface antigen in batches of each of these plasma components followed the introduction of screening of blood donors for this antigen. The examination for hepatitis B surface antigen by the most sensitive techniques of each unit of plasma used for fractionation should reduce further the contamination of these products with hepatitis B virus (see also WHO Reports, 1973, 1975, 1977). Hoofnagle *et al.* (1976) stressed that, nevertheless, fibrinogen, antihaemophilic factor and Factor IX remain "high-risk" hepatitis products particularly since it is becoming apparent that there is a hepatitis virus other than types A and B for which there are as yet no specific laboratory tests.

Craske *et al.* (1978) published the results of a retrospective survey of transfusion hepatitis associated with a brand of commercially prepared Factor VIII which was carried out in 1974–1975 in 24 centres for the treatment of haemophilia in the United Kingdom. Two types of hepatitis were observed: a short incubation non-B hepatitis, with an incubation period of 8–60 days, clinically identical with hepatitis A, but not associated with secondary cases, and hepatitis B, with incubation periods

of 50–185 days. Seventy-eight cases of hepatitis affecting 66 (17·7%) out of 371 patients transfused with one brand of Factor VIII were identified. Of these, 48 were non-B hepatitis and 30 were hepatitis B. Twelve patients contracted two attacks of hepatitis, non-B hepatitis followed by hepatitis B. Of the 48 cases of non-B hepatitis, seven were anicteric and most were clinically mild. Eleven of the 30 cases of hepatitis B were asymptomatic. One patient died in the acute stage of illness and it is thought that hepatitis B partially contributed to his death. Five out of the 30 patients became persistent carriers of the surface antigen for at least 1 year. The original concentrates of four of the six batches associated with cases of hepatitis B were found to contain hepatitis B surface antigen by radioimmunoassay. It was also noted that the reported incidence of hepatitis in patients with haemophilia in the United Kingdom before the introduction of commercial Factor VIII concentrate was about 1·8%. In 1978, Craske et al. tabulated the evidence for the existence of at least two types of Factor VIII-associated non-B transfusion hepatitis.

Alter et al. (1978) induced biochemical and histological hepatitis in five chimpanzees, using plasma or serum from four patients with acute or chronic non-A : non-B post-transfusion hepatitis and from a blood donor implicated in two cases of post-transfusion hepatitis. Tabor et al. (1978) transmitted non-A : non-B hepatitis to chimpanzees using serum from a patient with chronic hepatitis, whose blood caused acute hepatitis in a nurse following accidental inoculation with a needle, and serum from two former blood donors who transmitted clinical hepatitis to recipients. The third successful transmission of non-A : non-B hepatitis to chimpanzees was reported by Hollinger et al. (1978). The incidence of non-A : non-B hepatitis infection at four centres in the U.S.A. participating in the Transfusion-transmitted Viruses Study ranges from 5·4 to 18·5%. Sera from three blood donors who were involved in two cases of non-A : non-B hepatitis and sera collected from recipients of blood at least 12 days before clinical illness induced the infection in chimpanzees.

Wyke et al. (1979) described six cases of non-A : non-B hepatitis in London which followed administration of four different batches of concentrates of coagulation Factor IX from commercial and non-commercial sources. Of 17 patients who received Factor IX on account of chronic liver disease, four developed hepatitis, and in three of these the illness proved fatal. The incubation periods ranged from 42 to 103 days with a mean of 65 days. Three young male chimpanzees were inoculated with the same batch of Factor IX used for the treatment of the above patients,

another commercially prepared batch on which there were no reported adverse reactions, and documented infective non-A : non-B plasma. All three chimpanzees developed acute hepatitis after 10 weeks incubation. As in the patients, viral markers for hepatitis A and B, cytomegalovirus and EB virus were unchanged. It was concluded that until blood donors can be specifically screened for the virus(es) of non-A : non-B hepatitis, it would seem wise to restrict the use of both commercial and non-commercial blood concentrates to life-threatening situations. In particular, their use in patients with chronic liver disease should be avoided, since the risk of a resulting serious infection appears to be increased.

Bradley *et al.* (1979) transmitted non-A : non-B hepatitis to four chimpanzees by the infusion of three batches of Factor VIII (anti-haemophilic factor) implicated in hepatitis in two human recipients. Acute phase plasma from one of the infected chimpanzees induced hepatitis in two other chimpanzees after a relatively short incubation period of 3 weeks. Inoculation of a preparation of purified virus particles obtained from an open liver wedge biopsy resulted in hepatitis in two other chimpanzees after a shorter incubation period of about 15 days (see also Chapter 17). Cross-challenge experiments in chimpanzees should indicate whether one or more viruses are responsible for non-A : non-B hepatitis.

LABORATORY MARKERS FOR NON-A : NON-B HEPATITIS

At the time of writing, there is only one report by Shirachi *et al.* (1978) on the identification of specific antigen in patients with non-A : non-B hepatitis. The antigen was identified by double immunodiffusion assays between acute and convalescent sera from patients with post-transfusion hepatitis in Japan. The antigen was designated as hepatitis C antigen and it was found in the acute phase sera of all 13 patients with non-A : non-B hepatitis with an average long-incubation period of 7·2 weeks and a long duration of illness characterized by elevation of alanine amino-transferase levels for up to 17·5 weeks. The antigen was found in four out of ten acute phase sera obtained from patients with a type of non-A : non-B hepatitis characterized by an average incubation period of 5·7 weeks and abnormal alanine aminotransferase levels for an average of 5·8 weeks. The antigen was also detected in two out of 16 single serum specimens obtained during acute hepatitis from patients who had not been trans-

fused. Preliminary characterization of this antigen indicated that it migrated in the serum β-globulin fraction, it had a buoyant density of $1 \cdot 30$ g cm^{-3} in caesium chloride and a molecular weight of 100 000–300 000. Antibodies to this antigen were found in only 30% of the longer incubation type of non-A : non-B hepatitis and the antibodies were found in serum only transiently. Confirmation of these findings is awaited.

Reference has also been made to the identification of virus particles measuring 25–30 nm in diameter by immune electron microscopy of an open liver wedge biopsy obtained during the acute phase of non-A : non-B hepatitis from a chimpanzee inoculated with Factor VIII implicated in hepatitis in two human recipients (Bradley *et al.*, 1979). The incubation period of the hepatitis was about 3 weeks. Identification and purification of the viral antigen should lead to the development of specific laboratory tests for this form of hepatitis.

19

Hepatitis viruses : tissue and organ culture studies

The development of suitable cell and organ culture techniques for the isolation and propagation of the human hepatitis viruses continues to elude numerous investigators in many laboratories, although techniques are now available for obtaining primary cultures of differentiated human embryo hepatocytes, for preparing a semicontinuous human diploid liver cell line and for cultivating adult liver tissue (reviewed by Zuckerman, 1975, e, f). More recently, Tsiquaye et al. (1978a) also described the long-term cultivation of functionally active human embryo hepatocytes.

The detection of markers of hepatitis B virus infection by immunofluorescence and electron microscopy in primary human embryo liver cell cultures, in cultivated fragments of liver biopsy and in embryo liver organ cultures has been reported from several laboratories (Brighton et al., 1971; Coyne et al., 1971; Smith and Francis, 1972; Noyes, 1973; Shikata, 1973; Zuckerman and Earl, 1973; Kupradze et al., 1973; Ananiev et al., 1974; Watanabe et al., 1976; Gitnick, 1977), but serial replication of a transmissible agent has not been demonstrated. Some of these reports are briefly reviewed.

Noyes (1973) demonstrated by immunofluorescence the development of hepatitis B antigen in cultured human embryo hepatocytes after the inoculation of four different acute phase hepatitis B antigen-positive sera. The antigen was detected initially as irregularly shaped granular deposits in the nuclei of the liver cells 2 weeks after inoculation, and in the cytoplasm 4 weeks after exposure. After 5–6 weeks, foci of antigen-containing cells were found in the cultures and these foci gradually increased in size. The antigen was found only in the hepatocytes. Zuckerman

and Earl (1973) demonstrated by immunofluorescence hepatitis B core
antigen in the nuclei of cultured human embryo liver cells and surface
antigen in the cytoplasm of the cells. Similar fluorescence was observed in
the absence of cytocidal changes in cultured liver cells inoculated with the
supernatant fluid passaged in culture three times. Shikata (1973) demon-
strated hepatitis B antigen by immunofluorescence for 4 weeks after
inoculation of human embryo liver organ cultures with a serum obtained
from a patient with acute hepatitis B and antigenaemia. The antigen was
detected in hepatocytes 1 week after inoculation, and in the cytoplasm of
spindle-shaped cells which grew out from the original liver tissue.
Ananiev et al. (1974, 1976) reported the localization by immuno-
fluorescence of hepatitis B antigens in inoculated fragments of human
embryo tissue and human embryo kidney cells cultivated on the chorio-
allantoic membrane of the developing chick embryo. The antigen was also
found in the mesonephrons of the chick embryo. This effect was regularly
observed in 11 serial passages. Neutralization was carried out with human
hepatitis B antiserum.

The surface antigen has been produced, albeit infrequently, in human
embryonic liver organ cultures. A progressive rise in the titre of antigen,
measured by several techniques, has been demonstrated with a limited
number of sera (Zuckerman et al., 1972) but serial passage has not been
achieved. Watanabe et al. (1976) determined conditions required for the
establishment of human embryonic hepatocytes by organ culture tech-
niques using Rose's circumfusion chamber. In addition, another cell type
was found floating in the culture medium. These floating cells, referred
to as "oval cells", are probably immature hepatocytes. These cultures
were inoculated with two known infective hepatitis B sera and with other
sera containing hepatitis B surface antigen. Specific progressive immuno-
fluorescence was detected in the inoculated "oval cells"; 3 days after
infection antibody attachment was limited to the perinuclear membrane
and on the fifth day the cytoplasm was filled with fluorescent hepatitis B
surface antigen material. It was estimated that 20% of the "oval cells"
were synthesizing the surface antigen. Seven days after infection there was
evidence of destruction of the "oval cells". Clusters of more typical
hepatocytes which were attached to the bottom of the culture dish were
also synthesizing the surface antigen but this was limited to about one in
1000 cells. The immunofluorescent effect was demonstrated in up to four
passages, using the supernatant culture medium. It was estimated, using
solid-phase radioimmunoassay, that there was a 2- to 4-fold increase in the

titre of the surface antigen in the medium. In a later report, 12 passages were achieved with one inoculum with a resulting end-point titre corresponding to 10^{-14} of the original inoculum (Ishida *et al.*, 1978).

Panouse-Perrin *et al.* (1973) described preliminary results on the cultivation of hepatitis B virus in a human diploid cell line derived from open liver biopsies. Particles considered to be similar to hepatitis B core antigen were found by electron microscopy in the cells and in the supernatant fluid of cultures after serial passage. Cellular degeneration occurred after 14 passages and particles similar to the three morphological entities generally associated with hepatitis B were visualized. Serological tests, including radioimmunoassay, carried out on supernatant fluid obtained after ultrasonic disintegration of the cultures proved negative for the surface antigen; but precipitin lines were obtained by counter-immunoelectrophoresis when the fluids were tested against sera from patients in the early phase of convalescence from hepatitis B infection. Further details have been provided by Panouse-Perrin *et al.* (1975), including results obtained with conjunctival tissue, human embryo fibroblasts and KB cells. Interpretation of the electron micrographs and the serological findings is difficult (Zuckerman, 1975f). However, the specificity of immunofluorescent localization of the core antigen and surface antigen in the cultured cells suggests results similar to those obtained by several other investigators.

Clinical, pathological and experimental data indicate that the liver is the site of replication of hepatitis A and B viruses, and the failure to propagate these viruses in liver cells *in vitro* prompted Brumpt *et al.* (1978) to investigate whether in the case of hepatitis B excess viral coat protein, represented by the surface antigen, could interfere with the adsorption of the active virus by receptor blockage.

Zuckerman and Howard (1978) discussed the mechanism of virus attachment and penetration. Once a virus has come into contact with a susceptible host cell, the efficiency of infection is dependent on the ability of the virus to adsorb to specific recognition sites. It is possible that in the liver, virus particles in a sinusoid cannot enter the hepatocyte except by passing first through a macrophage. Zuckerman and Howard (1978) postulated that successful infection with the hepatitis viruses is dependent upon recognition of the virus by at least two different cell types, the macrophage and the hepatocyte. Virus adsorption is a function of the outer coat of the virus protein, in this instance hepatitis B surface antigen. However, an unusual and frequent feature of hepatitis B serum is the gross

excess or surplus of coat protein which conceivably could block the virus receptors. The fact that hepatitis B surface antigen did not block adsorption of several representative RNA and DNA viruses (Brumpt *et al.*, 1978) is interesting and clearly not due to inadequate titre of virus since the sera used were estimated to contain 10^{10} particles ml^{-1}. This apparent anomaly may be explained in a number of ways: hepatitis B virus may not share common receptors with any of the six indicator viruses used; the surface antigen may not contain that moiety responsible for the adsorption of the complete virus; and the cells used may not have appropriate surface receptors although qualitative adsorption and elution of hepatitis B surface antigen has been clearly demonstrated with these cell types in earlier studies. However, it should be noted that the concept of receptor mapping (Lonberg Holm *et al.*, 1976) is limited to a certain number of cell types, depends on their physiological condition and state of differentiation, and does not apply to every type of virus. In fact, the enveloped viruses have a different mode of adsorption and penetration.

Another possibility explored by Brumpt *et al.* (1978) was that excess surface antigen could interfere with the adsorption of the complete virus by the induction of interferon. Interference assays were used with representative RNA and DNA viruses. Direct interference with the challenge viruses was studied by plaque and cytopathic effect reduction methods under a variety of conditions of antigen adsorption. Interferon induction was also studied using whole sera containing hepatitis B virus and purified virus particles as inducer. Potentiation of interferon production was also attempted by priming and superinduction. However, no evidence of interferon induction *in vitro* was obtained. There was no response to priming of cultures with interferon either with Sendai virus or hepatitis B serum and antigens. With superinduction, a clear response was noted with Poly I : Poly C but not with hepatitis B surface antigen and hepatitis B virus particles. It seems, therefore, that neither sensitivity to nor induction of interferon can explain the failure to propagate the virus in cell culture systems *in vitro*.

Attempts to propagate hepatitis A virus and the newly identified hepatitis virus(es), the non-A : non-B agents, in cell and organ cultures have been unsuccessful. The development of suitable cell and organ culture techniques for the isolation and propagation of the hepatitis viruses thus remains a matter of urgency and high priority.

More recently, Provost and Hilleman (1979) reported the successful propagation of the CR 326 strain of human hepatitis A virus in primary

explant cell cultures of marmoset livers and in a normal foetal rhesus monkey kidney cell line (FRhK6). The virus was identified by a variety of techniques, including immunofluorescence, serum neutralization, immune adherence haemagglutination, radioimmunoassay, immune electron microscopy and by inoculation of susceptible marmosets. Cyto-pathic effects were not observed and the virus propagated more readily in the FRhK6 cell line. This opens the way to the detection and assay of hepatitis A virus *in vitro* and eventually to the preparation of hepatitis A vaccines.

Immunization against hepatitis

The development of specific serological tests for markers of infection with the viruses of hepatitis A and hepatitis B provided the means for investigating passive and active immunization. Specific tests for the newly identified form(s) of viral hepatitis (non-A : non-B infection) are not available at the time of writing.

HEPATITIS A

Passive immunization with immunoglobulin
Normal or ordinary human immunoglobulin, a 16% solution in a dose of 0·02–0·12 ml (kg body weight)$^{-1}$, administered intramuscularly before exposure to hepatitis A infection or early during the incubation period, prevents or attenuates a clinical illness (World Health Organization, 1964). The precise mode of action of immunoglobulin is not entirely clear but it is presumed that some degree of immunity against hepatitis A is obtained by globulin prepared from large pools of plasma. An apparent or sub-

clinical hepatitis which may follow active-on-passive immunity may lead
to prolonged immunity. The value of normal immunoglobulin for con-
trolling outbreaks of hepatitis A in situations such as infection in a
nursery school or semiclosed communities in large institutions and in the
family setting has been repeatedly demonstrated. However, the view has
been expressed that the very large scale and repeated use of immuno-
globulin in the general school-age population, as practised in some coun-
tries, is undesirable because unrecognized anicteric or subclinical cases
may disseminate the virus in the community. Indeed, evidence is accumu-
lating that excretion of virus in faeces may occur in such circumstances.
In addition, the practice appears to be wasteful and repeated injections of
immunoglobulin may be undesirable in, for example, healthy children
(World Health Organization Report, 1976). The availability of tech-
niques for titrating antibodies to hepatitis A provides a means of assay of
antibodies in normal immunoglobulin prepared from large pools of plasma
and studies are now in progress to determine the titre of antibody required
for protection and to establish the rate of clearance of passively acquired
antibody.

Active immunization
Hepatitis A virus has not been propagated to date in tissue culture. Once
this major step has been accomplished it should then be a relatively easy
matter to produce a conventional inactivated or an attenuated live vaccine.
    It was recently demonstrated experimentally that partially purified
hepatitis A virus from the liver of a rufiventer marmoset infected with the
CR 326 human strain and inactivated with formalin was immunogenic
when inoculated subcutaneously and protected other marmosets against
infection when challenged with live hepatitis A virus (Provost and
Hilleman, 1978). It is difficult to envisage the application of such an
approach to human immunization, even if it was feasible to purify virus
from non-human primate sources or from human faeces in sufficient
amounts to prepare adequate batches of experimental vaccines to undertake
safety and other test procedures.

HEPATITIS B

Passive immunization with immunoglobulin
The development of sensitive assays for hepatitis B surface antibody
allowed the selection of high titre plasma for the preparation of hepatitis B

immunoglobulin for clinical trials in order to determine the safety and efficacy of passive immunization. Many different batches of hepatitis B immunoglobulin have been tried in different countries using various titres of surface antibody determined by several techniques of different sensitivities and employing widely differing doses, schedules and circumstances. The results were frequently inconclusive or conflicting (reviewed by Zuckerman, 1975g; World Health Organization Expert Committee on Viral Hepatitis, 1977; Maynard, 1978). Many of the studies were faulty in some respects, notably the absence of randomized control groups given inert placebo preparations. Normal immunoglobulin prepared from plasma pools often containing small amounts of surface antibody was used as the control preparation in many of the trials, and this led to difficulties in interpreting the results because of the apparent effectiveness of such preparations for the prophylaxis of hepatitis B under some circumstances (Alter *et al.*, 1975).

There are many possible applications of hepatitis B immunoglobulin or normal immunoglobulin (containing a low titre of hepatitis B surface antibody) for the prophylaxis of hepatitis B infection, but these fall into three broad categories:

1. Post-exposure prophylaxis
2. Pre-exposure and repeated prophylaxis for continued exposure
3. Therapeutic.

Post-exposure prophylaxis with immunoglobulin. From the available data it seems that hepatitis B immunoglobulin is most clearly indicated for post-exposure prophylaxis after a single acute exposure to hepatitis B virus which occurs in accidental percutaneous inoculation (needle prick or "needle stick") or oral ingestion of clinical material containing markers of hepatitis B virus (Grady and Lee, 1976; Seeff *et al.*, 1975, 1978; Iwarson *et al.*, 1977).

The role of passive immunization with hepatitis B immunoglobulin for the protection of infants whose mothers are infected with hepatitis B virus is still not established and its protective effect has not yet been proved (Fawaz *et al.*, 1975; Cossart and Cohen, 1976), but on the whole the number of infants treated has been very small. However, Tamburro and Leevy (1973) noted that none of 30 newborn infants of mothers with hepatitis B surface antibody and detectable antibody in the cord blood developed hepatitis during a period of observation extending from 3 months to 2 years, despite living in an environment with high prevalence of narcotic drug addiction and hepatitis B. The results were interpreted as

evidence that transplacental transfer of hepatitis B surface antibody provides passive immunity to infection. This, of course, would be difficult to attain by the administration of hepatitis B immunoglobulin to antigen carrier mothers, but the observations provide a rationale for passive immunization of the newborn to a carrier mother.

The following year, Kohler *et al.* (1974a) reported that five out of six infants born to mothers who contracted hepatitis B during pregnancy subsequently developed evidence of infection with this virus within 5 to 12 weeks of birth, whereas four such babies treated with immunoglobulin or plasma containing hepatitis B surface antibody remained antigen-negative for 5 to 14 months. There are several other similar individual case reports. A more comprehensive randomized double blind placebo controlled trial of hepatitis B immunoglobulin was carried out by Beasley and Stevens (1978) in an attempt to reduce the frequency of vertical transmission in Taiwan. The infants received a single dose of one of three preparations, hepatitis B immunoglobulin, normal immunoglobulin without detectable hepatitis B surface antibody or heat-treated albumin.

The most striking effect of hepatitis B immunoglobulin was a later onset of antigenaemia in the infants. There was also a slight but statistically insignificant lower rate of antigenaemia during the first year of life in the babies receiving hepatitis B immunoglobulin, and a larger number of the babies in this group developed active antibodies at 10 months of age or later. However, infants who received hepatitis B immunoglobulin within the first 48 h of birth developed significantly fewer persistent infections. These results indicate that there is a need to determine the effect on perinatal transmission of a larger single dose of hepatitis B immunoglobulin at birth and repeated doses of immunoglobulin.

Pre-exposure and repeated prophylaxis with immunoglobulin. Marginal to significant differences have been found during studies of persons exposed to hepatitis B virus acutely or continually, for example in haemodialysis units, when preparations of hepatitis B immunoglobulin or ordinary immunoglobulin containing a low titre of hepatitis B surface antibody were used (see, for example, Szmuness *et al.*, 1974; Desmyter *et al.*, 1975; Prince *et al.*, 1975a; Redeker *et al.*, 1975; Delons *et al.*, 1976; Prince *et al.*, 1978a). In view of the temporary protective effect of passive immunization by any immunoglobulin preparation, however, it is debatable whether it should be used at all under conditions of chronic exposure, since multiple injections would be needed until either active immunity deve-

loped or the individual left the endemic situation. In the case of haemo-
dialysis units in particular, other preventive measures employing good
hygienic precautions and established principles of epidemiological practice
have been used to control hepatitis B with considerable success, so that
passive immunization in such settings should probably be reserved for
circumstances in which control by other measures cannot be effectively
implemented. It may then be possible to achieve passive-active immunity
by using immunoglobulin containing a relatively low titre of hepatitis B
surface antibody. However, in due course the preferred approach in
endemic settings will be by active immunization, when a safe and
effective hepatitis B vaccine becomes available.

Therapeutic use of immunoglobulin. Immunotherapy with hepatitis B immuno-
globulin has been used for chronic liver disease associated with persistent
hepatitis B antigenaemia and acute fulminant hepatitis B. Reed *et al.*
(1973a) investigated the effects of infusion of hepatitis B immunoglobulin
in six patients with chronic active hepatitis, in five of whom the surface
antigen was persistently detected in the serum. The intravenous infusions
were well tolerated, and although immune complexes were detected in the
circulation, there was minor and transient evidence suggestive of an
immune complex reaction in two patients. Clearance of the antigen was
achieved for up to 9 days in two patients with low initial titres of antigen.
However, unless the liver can be cleared of the virus, long-lasting benefits
are unlikely to be attained by such treatment.

The supervention of hepatic encephalopathy in acute viral hepatitis
indicates a very poor prognosis; for example, Trey (1972) reported that
142 (79·8%) of 178 patients with viral hepatitis who progressed to stage
IV coma died. More recently, the use of intravenous hepatitis B immuno-
globulin for the treatment of fulminant hepatitis B was evaluated in 30
centres in the United States. The intravenous preparation was prepared
from immunoglobulin for intramuscular use by treatment with plasmin.
Human albumin, free from surface antibody, was used as placebo. Two
doses of hepatitis B immunoglobulin were used, 1·32 and 5·28 g of IgG
protein. There was no significant difference between the two groups or
between the group of patients treated with immunoglobulin and the group
receiving the placebo. Thus, treatment with exogenous hepatitis B surface
antibody was of no value (Acute Hepatic Failure Study Group, 1977).

The World Health Organization Expert Committee on Viral Hepatitis
(1977) recommended the following guidelines for passive immunization
against type B hepatitis:

1. The major indication for hepatitis B immunoglobulin is post-exposure prophylaxis after a single acute exposure to hepatitis B virus, such as when blood known or strongly suspected to contain hepatitis B surface antigen is accidentally inoculated ("needle stick"), ingested orally (as in a pipetting accident), or splashed on to mucous membranes. Hepatitis B immunoglobulin with a high titre of surface antibody, standardized against a reference preparation, should be administered in a dose of approximately 5 ml for adults as soon as possible after such exposure.

2. In endemic settings such as haemodialysis units where hepatitis B virus transmission is known to occur and where preventive hygienic measures cannot be implemented, prophylaxis with immunoglobulin containing surface antibody for susceptible staff may be considered on a continuing basis until transmission of hepatitis B virus can be abolished. There is at present some controversy whether immunoglobulin with low titre or high titre hepatitis B surface antibody is preferable and what the dosage and frequency of administration should be under these circumstances.

3. Individuals with a significant titre of hepatitis B surface antibody are generally resistant to hepatitis B infection and usually require no form of passive immunization against it.

4. Passive immunization with hepatitis B immunoglobulin is not indicated after blood transfusion, provided that hepatitis B surface antigen-positive blood has been excluded by sensitive methods, because under such circumstances most cases of post-transfusion hepatitis are not due to infection with hepatitis B virus.

In the context of the latter statement it is interesting to note that in a small study of post-transfusion hepatitis in patients undergoing cardiac surgery, ten cases of chronic liver disease were identified among 44 patients with acute non-A : non-B hepatitis. It was also observed that the administration of immunoglobulin (containing a high titre of surface antibody or commercially available immunoglobulin) before operation to these patients, who received large amounts of blood, reduced the incidence of post-transfusion hepatitis and the incidence of chronic liver disease associated with non-A : non-B hepatitis (Knodell et al., 1976, 1979). These important observations should be confirmed and extended.

Active immunization

A vaccine against hepatitis B is needed for groups which are at an increased risk of acquiring this infection. These groups include individuals requiring repeated transfusions of blood or blood products, prolonged inpatient treatment, patients who require frequent tissue penetration or need repeated access to the circulation, patients with natural or acquired immune deficiency and patients with malignant diseases. Viral hepatitis is an occupational hazard among health care personnel and the staff of institutions for the mentally retarded. High rates of infection with hepatitis B occur in drug addicts, homosexuals and prostitutes. Persons working in high endemic areas are also at an increased risk of infection. Women in areas of the world where the carrier state in that group is high, are another segment of the population requiring immunization in view of the increased risk of transmission of the infection to their offspring. Consideration will also have to be given to persons living in certain tropical and subtropical areas where present socio-economic conditions are poor and the prevalence of hepatitis B infection is high.

The repeated failure to cultivate and passage hepatitis B virus serially in tissue or organ cultures (Zuckerman and Earl, 1973; Zuckerman, 1975d) has hampered progress towards the development of a conventional vaccine. Attention has therefore been directed towards the use of other preparations for active immunization against hepatitis B (see, for example, Zuckerman and Howard, 1973, 1975; Zuckerman, 1975h; Krugman, 1975; Melnick *et al.*, 1976).

Active immunization was attempted by Krugman *et al.* (1970, 1971) and Krugman and Giles (1973) using as the immunogen a well-documented infective human serum (MS-2 serum; see Fig. 20.1) that contains hepatitis B virus (Krugman *et al.*, 1974). The serum was diluted 1 in 10 in distilled water and heated at 98°C for 1 min. The serum treated in this manner was not infective and it successfully prevented or modified hepatitis B in 69% of susceptible persons inoculated with the heated serum and challenged with the original infective serum 4–8 months later. The results with the heat-inactivated serum were essentially the same after one, two or three inoculations. In other studies on active immunization, Soulier *et al.* (1972) used serum containing hepatitis B surface antigen obtained from a healthy carrier and heated at 60°C for 10 h, but the virus was not completely inactivated as shown by the acquisition of antigen and elevated serum aminotransferase levels in a proportion of the recipients. In any case, the use of heated whole serum is a somewhat crude way of
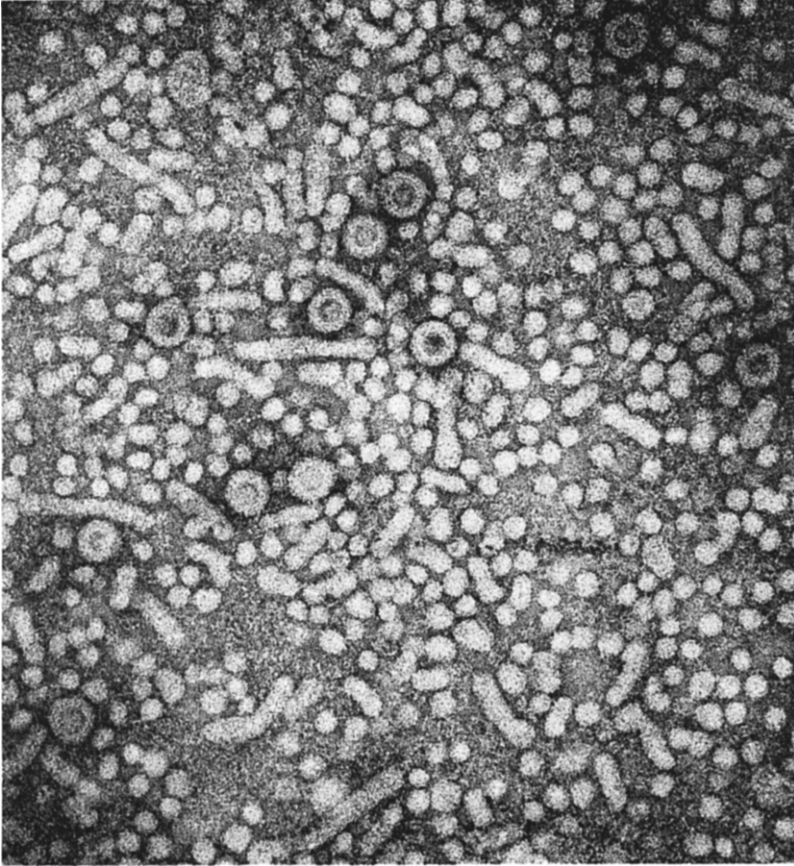
FIG. 20.1. Electron micrograph of MS-2 serum showing the three morphological entities of hepatitis B: small pleomorphic spherical particles; tubular forms; and the complete hepatitis B virus, mainly the double-shelled form. × 126 000. (From a series by S. Krugman, R. G. Bird and A. J. Zuckerman.)

inducing immunity and it is unlikely to be licensed for general use. Nevertheless, the work of Krugman and his associates laid the foundations for the subsequent development of unconventional subunit hepatitis B preparations which could be used as vaccines.

Subunit small particle hepatitis B vaccines
Since the separated viral coat material, in the case of hepatitis B the surface antigen, leads to the production of protective surface antibody as

shown in serological surveys and experimental studies, the possibility of using purified 20–25 nm (average diameter 22 nm) spherical hepatitis B surface antigen particles is attractive. Such experimental vaccines (Fig. 20.2) have been prepared from the plasma of apparently healthy carriers of the surface antigen (Purcell and Gerin, 1975, 1978; Hilleman et al., 1975, 1978; Maupas et al., 1976, 1978; Prince et al., 1978b; Tao et al., 1978).

The preparation of such a vaccine for use in human beings from human viral antigens not grown in cell culture, but obtained from infected persons, is an entirely new approach in preventive medicine.

Although it is generally accepted that the 22-nm hepatitis B surface subunit preparations when pure are free of nucleic acid and therefore non-infectious, the fact that the starting material for their preparation is human plasma obtained from persons who are persistent carriers of the surface antigen, which is a marker of hepatitis B virus, means that extreme caution must be exercised to ensure their freedom from all harmful con-taminating material. Some concern has been expressed on the possible induction of harmful immunological reactions to host components, in-cluding pre-existing structures of liver cells, which may be present either as an integral component of the surface antigen or be intimately associated with the antigen as a contaminant (Zuckerman, 1975h, 1976b; Melnick et al., 1976) but reactions of this type have not been observed so far with the highly purified experimental vaccines when tested for safety in chim-panzees (Prince et al., 1978b). The absence of such autoimmune response in chimpanzees is reassuring, although of questionable relevance, since the chimpanzee may be an inappropriate model for such markers.

Human hepatitis B virus has been successfully transmitted to chim-panzees (Chapter 17) and although the infection is mild the biochemical, histological and serological responses in these primates are very similar to those in humans. The relative susceptibility of humans compared with chimpanzees for developing hepatitis B infection is not known, and it cannot be measured for obvious reasons, but in general terms chimpanzees are highly susceptible. Sensitive tests for surface antigen, core antibody, surface antibody, DNA polymerase and for hepatitis B e antigen and anti-e are available and these provide the means of monitoring vaccines. The 22-nm particle vaccines which were treated with formalin were found to be safe in a number of susceptible chimpanzees, and the vaccines were protective as shown by challenge of the chimpanzees with infectious material (Buynak et al., 1976a, b; Purcell and Gerin, 1978).
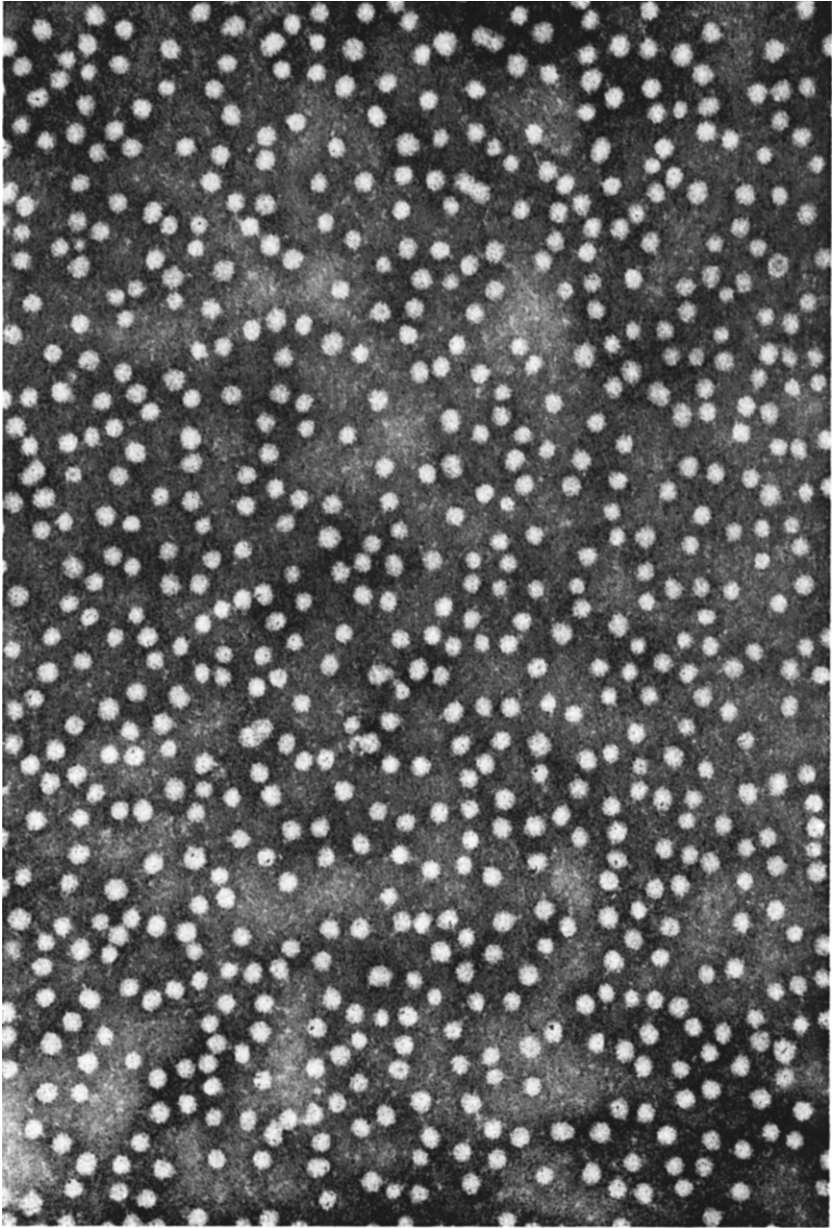
FIG. 20.2. Purified preparation of 20–25 nm spherical hepatitis B surface antigen. Such preparations are being evaluated as subunit small particle hepatitis B vaccines. × 126 000.

The results of the first human trial with an experimental subunit 22-nm particle vaccine were reported by Maupas et al. (1976, 1978). In this study immunoadsorbent columns containing, respectively, hepatitis B surface antibody of human origin and antibodies of animal origin to human serum have been used for the partial purification of the surface antigen from the plasma of carriers for use as an experimental subunit hepatitis B vaccine. After inoculation of this material in chimpanzees, it was administered to the patients and staff of several haemodialysis units in France. The study did not include a control group, but a marked difference in the incidence of hepatitis B infection was observed subsequently between the immunized group and a group of patients and staff who had not received the vaccine. Abnormal autoimmune markers were not detected among the immunized group.

Hilleman et al. (1978) prepared several batches of highly purified 22-nm spherical particle vaccine which were inactivated with formalin. These vaccine preparations elicited specific antibody responses in guinea pigs, mice and non-human primates. The vaccine was shown to be non-infectious in susceptible chimpanzees and protected chimpanzees against challenge with human hepatitis B virus. Small-scale studies in human volunteers with an aqueous preparation and with vaccine containing an alum adjuvant have shown that the vaccine is safe and that it elicited surface antibody in individuals with and without pre-existing homologous antibody. The vaccine is being used to boost the titre of surface antibody in plasma donors for the subsequent preparation of hepatitis B immuno-globulin. Trials to determine the prophylactic efficiency of this vaccine preparation in groups of individuals exposed to high risks of infection, such as patients and staff of haemodialysis units and young male homosexuals, are in progress.

The World Health Organization Expert Committee on Viral Hepatitis (1977) proposed certain minimum criteria and guidelines for the preparation of experimental hepatitis B vaccines. These include the following:

1. Consideration should be given to the selection of hepatitis B surface antigen-positive plasma which is devoid of hepatitis B virus particles, DNA polymerase and hepatitis B e antigen as the starting material from which the experimental vaccine is to be prepared since these components are associated with a relatively greater infectivity of hepatitis B virus. However, this association is not absolute, and the absence of these indicators does not guarantee lack of infectivity. A further problem is that these markers of infectivity are usually found

    in plasma samples with the highest titres of hepatitis B surface antigen, which are precisely those which are potentially the most useful for the preparation of vaccine.

2. The vaccine should be prepared in such a way as to remove most, if not all, possible contaminants such as liver antigens, nucleic acids and other host proteins.

3. To minimize the risk of introducing other agents into the vaccine pool, consideration should be given to preparing experimental vaccines from a small number of carefully selected donors who have undergone repeated plasmapheresis, without incurring adverse reactions in recipients of their plasma or plasma products. At present there are no tests for carriers of hepatitis viruses other than hepatitis A and hepatitis B.

4. Foreign antigens must not be introduced into the vaccine during the purification procedure. Experimental vaccines prepared by methods involving this risk must be rigorously examined for the presence of foreign proteins by the most sensitive techniques available.

5. The vaccine must be subjected to inactivation methods which are most likely to inactivate hepatitis B virus and which are reproducible and acceptable for use with other virus vaccines. Both heating (e.g. treatment at 60°C for 10 h) and formaldehyde solution at a concentration of up to $0 \cdot 5$ ml l^{-1} appear to fulfil these criteria, but under some circumstances heating did not inactivate hepatitis B virus completely, and exposure to formaldehyde solution is ineffective when viruses are aggregated or when contaminating proteins are present in high concentrations.

6. The method of vaccine preparation must be consistently demonstrated to inactivate or remove hepatitis B virus by appropriate safety tests in seronegative (i.e. susceptible) chimpanzees, and also be shown to yield a product which is free of other viruses, bacteria and mycoplasmas by appropriate *in vitro* and *in vivo* tests.

7. The preparation method should consistently yield a vaccine that is immunogenic and capable of protecting against challenge with live hepatitis B virus in chimpanzees. Eventually an appropriate immunogenicity extinction test in other laboratory animals may be substituted for this requirement if a good correlation can be demonstrated between the results of such a test and evidence of protection in chimpanzees.

8. Initial tests in human volunteers of any new experimental hepatitis B vaccine should be performed in a stepwise manner, starting with a

very small number of healthy adult volunteers capable of giving informed consent. The safety of the vaccine must be demonstrated in this initial group before it is administered to a larger group.

9. Recipients of experimental hepatitis B vaccines should be carefully monitored at frequent intervals for evidence of immune response to the vaccines, immune response to contaminating antigens, development of hepatitis, presence of autoimmune markers, and other untoward reactions. Provisions should be made for long-term evaluation of vaccine safety, particularly during the initial tests in human beings.

Additional safety considerations for the subunit hepatitis B vaccines

Reference has been made to the possible induction of harmful immunological reactions to host components which may be present in the purified 22-nm particle subunit vaccine preparations. It should also be remembered that some purified inactivated viral vaccines such as measles and respiratory syncytial virus vaccines have sensitized rather than protected the recipients. In terms of hepatitis B this may be important because it has been shown that there are similarities between certain features of hepatitis B and type III hypersensitivity reactions in which the severity of the disease may depend on the balance between antigen and antibody (Almeida and Waterson, 1969; Brzosko et al., 1971; Nowoslawski et al., 1972; Kater et al., 1974; Trepo et al., 1974; Woolf et al., 1976).

The safety of any viral vaccine must also be carefully considered in relation to its possible oncogenic potential. A number of viruses, particularly members of the herpesvirus group such as herpes hominis type 2 and EB virus (human herpes virus 4) have been implicated in the aetiology of certain human cancers. Difficulties have, for example, been met in the development of a cytomegalovirus (human herpes virus 5) vaccine since this is a member of the group, although it has not been implicated in neoplasms. Hepatitis B virus is an unclassified DNA virus and there is considerable evidence of a close association between this virus and primary hepatocellular carcinoma (reviewed by Szmuness, 1978; Zuckerman, 1978a). It was therefore suggested by Zuckerman (1978b) that before large-scale trials of the subunit hepatitis B vaccines are undertaken, particularly in young children in the developing countries (see, for example, Maupas et al., 1978), it is essential to ensure that any benefit that accrues from immunization is not outweighed by undesirable and potentially serious hazards. Fortunately, the necessary sophisticated technology to ensure the complete inactivation of any live virus or residual

DNA in such preparations derived from potentially oncogenic viruses is now available.

Hepatitis B polypeptide vaccines

Alternative vaccines are being prepared from the constituent polypeptides of the 22-nm spherical particles of the hepatitis B surface antigen. Vaccines prepared from such polypeptides should have an added margin of safety since they would be even less likely than the 22-nm particle subunit vaccines to contain infectious virus or contaminating host proteins that might lead to untoward reactions in some individuals.

Preparations consisting of separated 22-nm particles, which constitute the bulk of the hepatitis B surface antigen material in the sera of most carriers, have been analysed both chemically and serologically in several laboratories, but with varying results (reviewed by Howard and Burrell, 1976). Although in most studies at least two major polypeptides were found in the molecular weight range of 20 000 to 30 000, variable amounts of larger components were frequently present (see, for example, Howard and Zuckerman, 1974; Shih and Gerin, 1975; Dreesman et al., 1975; Shih and Gerin, 1977; Skelly et al., 1978). It is possible that some of these polypeptides represent integral host proteins which may play a role in maintaining and preserving surface antigenic reactivity.

Various studies have shown that individual polypeptides are immunogenic after inoculation into guinea pigs. Dreesman et al. (1975) prepared antisera to five polypeptides derived by solubilization of hepatitis B surface antigen with sodium dodecyl sulphate in the presence of urea. Some variation was found in the nature of the responses obtained against individual polypeptides prepared from subtypes adw and ayw. In each case, however, antibody to surface antigen was successfully raised in animals inoculated with the 24 000, 35 000 or 40 000 molecular weight polypeptides. Antisera to six or seven polypeptides, ranging in molecular weight from 23 000 to 97 000 separated from purified surface antigen particles of subtypes adw and ayw were raised in guinea pigs by Gold et al. (1976). Most or all of the isolated polypeptides stimulated antibodies to the d or y subdeterminants and therefore these antigenic determinants are part of the constituent structure of the polypeptides. It was concluded, therefore, that despite the range in molecular weights (23 000 to 97 000), the constituent polypeptides of hepatitis B surface antigen are similar in their virus-specific immunochemical structure (see also Peterson et al., 1977).

Shih and Gerin (1977) found up to seven polypeptide species in purified 22-nm particles representing the three major subtypes of the surface antigen (*adw*, *ayw* and *adr*). Two polypeptides with molecular weights 23 000 and 29 000 were found as the major components. The remaining polypeptides varied within each subtype both in number and in relative concentration. The major 29 500 molecular weight polypeptide and the minor 53 000 molecular weight component appeared to be glycoproteins. Peterson *et al.* (1977) identified two major bands of polypeptides, with molecular weights of 16 000 and 40 000–90 000. The purified poly-peptides from the 22 000 and 28 000 molecular weight bands had essentially identical amino acid composition to each other and to the intact surface antigen. The amino-terminal and carboxy-terminal sequences of amino acids in these two major polypeptides, which accounted for 75% of the total protein, showed that hepatitis B surface antigen with deter-minants *adw* consisted of a single major polypeptide chain or two homo-logous polypeptide chains that differed only in limited areas of their structure. The difference in the molecular weight of these two components is due to the carbohydrate moiety of the glycoprotein of the second band. Injection of the 22 000 molecular weight polypeptide emulsified in Freund's complete adjuvant elicited an antibody response in guinea pigs. Antibodies were produced to the group-specific determinant *a* and to one of the major subdeterminants of the surface antigen *d*. The 28 000 molecular weight polypeptide, however, did not induce an antibody response and it was suggested that the discrepancy with the immuno-genicity of the glycosylated peptides reported in other studies could have been due to contamination of the two major polypeptides.

Shih *et al.* (1978) later studied the immunogenicity of the major poly-peptides separated from purified surface antigen using double antibody radioimmunoprecipitation. Each of three polypeptides, 23 000, 29 500 and 72 000 molecular weights respectively, were trace-labelled and used as the radioligand in the assay. Each of these polypeptides contained both the group-specific determinant and the subtype-specific determinant as shown by precipitation by antiserum to the native surface antigen and by antisera prepared against the separated polypeptides, thereby indicating a high degree of serological relationship between these three components. The production of immunologically active hepatitis B surface antigen polypeptides reported above from various laboratories employed severe chemical conditions for component separation before further serological analysis. Exposure of antigenically reactive material to the anionic deter-

gent sodium dodecyl sulphate in the presence of a reducing agent, which is essential for efficient polypeptide analysis, invariably abolishes the affinity of proteins for homologous antibody which may only be recovered with difficulty. Furthermore, considerable losses in protein yield frequently occur when extraction of individual polypeptides is attempted from polyacrylamide gels.

In more recent studies, Skelly et al. (1978) resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis five major polypeptides from purified hepatitis B surface antigen. The molecular weights were 64 000 (P64), 32 000 (P32), 30 000 (P30), 28 000 (P28) and 23 000 (P23). The 28 000 (P28) molecular weight component was found to contain a type A carbohydrate chain after specific radiolabelling in a reaction employing the enzyme galactose oxidase. Further experiments with trace-labelled concanavalin A confirmed the glycosylated nature of this component. Additional glycoproteins were identified as a result of binding the labelled lectin and included components P30 and P32. The failure to identify previously the carbohydrate content of P30 and P32 by utilizing the enzyme galactose oxidase suggests the presence of a B-type carbohydrate moiety on these components containing mannose and glucose only. A carbohydrate moiety was not found to be associated with P23 by either of these techniques.

Non-ionic detergents and bile salts have been used extensively for the dissociation of viruses into soluble complexes which retain biological activity. Simons et al. (1973) disrupted the virus envelope of Semliki forest virus into soluble protein and lipid complexes by treatment with Triton X-100, an alkylpolyethoxy alcohol. The protein and lipid complexes were then separated by density gradient centrifugation in the presence of the detergent. Hayman et al. (1973) separated successfully the surface glycoproteins of influenza and mouse mammary tumour viruses by affinity chromatography using columns of immobilized phytohaemagglutinin equilibrated with buffer containing sodium deoxycholate. A considerable advantage in these studies was the retention of the biological activities of the envelope glycoproteins after disruption of the virus. Skelly et al. (1979) disrupted purified hepatitis B surface antigen with 2% Triton X-100 in the presence of salt to yield a product with an estimated sedimentation coefficient of 3·9 S. Fractionation of radiolabelled antigen by passage through columns of immobilized concanavalin A resulted in the separation of three components into two fractions. The first fraction, which did not bind to the lectin, contained exclusively a 64 000 molecular weight

polypeptide. This component reacted with serum albumin antibodies to produce peptide maps similar to albumin after treatment with trypsin. These and other experiments indicate the presence of serum albumin in purified preparations of hepatitis B surface antigen (see also Millman *et al.*, 1971; Neurath *et al.*, 1974). It is noteworthy, in this context, that the appearance of autoantibodies to albumin has been described as a feature of hepatitis B infection (Lenkei *et al.*, 1976, 1977) and, in addition, it has been suggested that surface antigen from individuals persistently infected with hepatitis B virus may bind to polymerized albumin (Neurath *et al.*, 1978b). The second fraction was obtained by eluting bound material from the immobilized lectin column with α-methyl-D-mannoside, and was found to contain the P28 and P23 polypeptides as the major components. Since P23 was not found previously to be glycosylated, this polypeptide probably remained bound to P28 by a protein–protein linkage after detergent treatment. The reactivity of this material with antibodies to the surface antigen, in the absence of any significant reactions with antibodies to normal serum components, indicates that hepatitis B surface antigen reactivity resides with this subunit preparation. Thus the technique of Triton X-100 solubilization followed by affinity chromatography described by Skelly *et al.* (1979) allows the preparation of milligramme quantities of immunologically reactive material which was not previously possible by preparative sodium dodecyl sulphate polyacrylamide gel electrophoretic techniques. Studies are now in progress to determine the immunogenicity of this material and to compare its efficacy with the 22-nm particle sub-unit vaccine for the protection of susceptible chimpanzees to hepatitis B infection. The preliminary results with P22 and P25 polypeptides reported by Hollinger *et al.* (1978), using the far less efficient sodium dodecyl sulphate polyacrylamide gel separation techniques for the peptides, are encouraging in this respect.

Additional factors for consideration in the design of hepatitis B vaccines
An aspect which is yet to be clarified is whether hepatitis B vaccines should contain multiple subtypes of hepatitis B surface antigen to afford maximum protection or whether vaccine prepared from a single subtype will uniformly protect against all subtypes of hepatitis B virus. Most experimental data obtained from cross-challenge experiments in chimpanzees suggest that infection with one subtype of hepatitis B virus affords substantial protection against other subtypes, but evidence for occasional reinfection among patients indicates that cross-protection may not be

complete. However, the several reports on the occurrence of concurrent hepatitis B surface antigen and heterotypic surface antibody (Duvillers et al., 1974; Tabor et al., 1977b; Moraczewska et al., 1978) may have implications for the monovalent 22-nm subunit hepatitis B vaccines which are now under trial. Although there are several possible explanations for these paradoxical combinations of surface antigen and heterotypic antibody, the existence of this phenomenon indicates the need to establish the extent of cross-protection by experimental hepatitis B vaccines against one or more subtypes and to determine whether a multivalent vaccine will be necessary, since more than one subtype of hepatitis B virus is endemic in most areas of the world (World Health Organization, 1977).

Another aspect for consideration in the design of a suitable immunogen is the possible need for stimulating an anti-*e* response (Neurath et al., 1976) and antibodies reacting with recently described specificities on the complete hepatitis B virus (Alberti et al., 1978). Neurath et al. (1976) reported that sera from one asymptomatic carrier of hepatitis B surface antigen, free of surface antibody but positive for anti-*e*, aggregated the hepatitis B virion but not the small spherical forms of the surface antigen and it was suggested that this was due to the presence of *e* determinants on the surface of the virion. Although these findings and interpretation have been disputed by other workers (Takahashi et al., 1978; Gerin et al., 1978), it is important to establish whether antibody to *e* might play any role in a protective immune response. Indeed, Prince et al. (1978b) mentioned preliminary observations indicating that a preparation of purified *e* antigen, free of detectable surface antigen, protected chimpanzees against challenge with live hepatitis B virus in the absence of surface antibody. The antibodies described by Alberti et al. (1978) did not correlate with anti-*e*, but it is possible that only one of the various *e* antigen determinants is involved. The presence of these latter precipitating antibodies may be relevant to the clearance of circulating hepatitis B virions and the termination of acute infection; and their absence in all but one of the patients with chronic active hepatitis might explain why the infection persists in such patients. These antibodies might also be of relevance in protective immunity.

Perhaps one of the most interesting prospects for the future is the development of synthetic vaccines (Sela, 1966, 1975, 1977). An immuno-chemical study of purified hepatitis B antigens is essential, and determination of the primary sequence of the haptenic peptide of the surface antigen may provide a way of developing a synthetic peptide, which could

serve as a suitable immunogen. Once detailed data are available on the protein, polypeptide and amino acid sequences and three-dimensional structure of hepatitis B surface antigen, it should be possible to define by animal immunization the moiety responsible for the antigenic activity (Rao and Vyas, 1973 ; Zuckerman and Howard, 1973 ; Zuckerman, 1975g; Melnick et al., 1976; Peterson et al., 1977; Peterson et al., 1978).

There are reports in the literature which support the feasibility of such an approach. For example, previous studies with tobacco mosaic virus showed that an antigenic determinant responsible for the immunogenic activity of the virus and its amino acid sequences can be identified (Stewart et al., 1966; Young et al., 1968). Such amino acid moieties can be synthesized and, when coupled to a carrier protein, will induce the production of neutralizing antibody in experimental animals (Fearney et al., 1971). Arnon et al. (1971) also showed that it was possible to use a synthetic macromolecule for eliciting antibodies reacting exclusively with a specific region of a native egg-white lysozyme. This was achieved by synthesizing a particular segment of the enzyme from its amino acid components, attaching the peptide to branched poly-DL-alanine as carrier and using the conjugate for immunization. The resulting antibodies to a completely synthetic immunogen reacted with native lysozyme via a unique region which is conformation-dependent. Of course, in the case of lysozyme both the amino acid sequence and the three-dimensional structure are known, and the synthesized peptide was designed on the basis of previous information concerning its contribution to the antigenic specificity of the molecule. More recently, Langbeheim et al. (1976) accomplished the neutralization with antibody to a synthetic antigen of a bacterial virus, coliphage MS-2 which is an RNA-containing virus with icosahedral symmetry.

As pointed out by Sela (1975, 1977) the conceptual way to synthetic vaccines is now open since if it can be done for one protein it should be possible to achieve this for other proteins. With the rapid advances in knowledge of protein chemistry and X-ray crystallography a similar approach could be used for at least some viral coat proteins and, in particular, with viruses such as the hepatitis viruses which cannot be cultivated in tissue culture. It is necessary, of course, to determine whether antibodies to such synthetic immunogens will be protective and whether the antibodies will persist. There are, however, many obvious advantages in attaining the ultimate goal of multivalent synthetic vaccines to replace current vaccines which often contain many irrelevant microbial

antigenic determinants, proteins and other material which contaminate the essential immunogen and which may lead to untoward side effects (see also Wilson, 1967; Zuckerman, 1975h, 1978c).

21 | Interferon and antiviral therapy in chronic hepatitis B infection

INTERFERON

Interferons are small glycoproteins that are able to inhibit the replication of animal viruses and are released after an appropriate induction in cells of animal species ranging from fish to mammals. All interferons are of molecular weights ranging from 12 000 to 160 000, depending on the host and the cell type used for their synthesis. Interferons inhibit the replication of a wide range of both DNA and RNA viruses, although the sensitivity of individual viruses to the action of interferon varies.

The primary site of action appears to be on the cell membrane where an interferon-specific receptor system is located. This system consists of a binding site made of gangliosides and an activator site probably consisting of glycoproteins. The integrity of the cell membrane is necessary for the induction of the antiviral effect, and an interferon-specific modification of the cell membrane components is the requisite primary step for anti-viral activity. The cells also produce new products such as a protein kinase and a nuclease, as well as other less precisely defined proteins.

Inhibition of newly synthesized virus-specific proteins is probably due to alteration of the methylated cap of an RNA molecule and inhibition of synthesis of polypeptides at the ribosomal level. Modification of RNA

and transfer-RNA may occur in cells treated with interferon and, with large amounts of interferon, overall cellular RNA and protein synthesis may also be depressed.

In addition to their antiviral effect, interferons can decrease cell replication and inhibit tumour growth, inhibit the replication of certain intracellular organisms such as rickettsiae and protozoa, alter the sensitivity of cells to toxins and hormones, and inhibit circulating antibody formation and cell-mediated immunity as well as enhance phagocytosis and cytotoxicity of lymphocytes (reviewed in a World Health Organization Memorandum, 1978).

In general, cells make little interferon and for only a short period of time. Therefore, very large numbers of cells are required for production of interferon for clinical use. At present, three main sources of human interferon are available: leucocytes, human diploid fibroblasts and transformed human lymphoblastoid cells. Most clinical studies have been carried out with human leucocyte interferon and more recently diploid fibroblast interferon has been used. These interferons can be distinguished by differences in their physicochemical properties, antigenic structure and in their stability and host specificities.

Recent reports indicate that the administration of human interferon both in humans and in chimpanzees has an inhibitory effect on replication of hepatitis B virus. Changes were transient when leucocyte interferon was given for less than 2 weeks but when a 4-week or longer course was given to two patients with chronic active hepatitis, marked falls occurred in hepatitis B virus-associated DNA, in DNA polymerase activity, and in core antigen levels which were maintained for up to 15 weeks after stopping treatment (Greenberg et al., 1976). A synthetic interferon inducer (Purcell et al., 1976) produced similar changes in chimpanzees. The effect of human leucocyte interferon on markers of hepatitis B virus in carrier chimpanzees was dramatic but transient (Zuckerman et al., 1978). With fibroblast interferon, Desmyter et al. (1976) observed reduction in hepatitis B surface antigen and core antigen within liver tissue in a patient with chronic hepatitis and in chimpanzees, while Kingham et al. (1978) reported a marked fall in titre of core antibody in two patients, which was accompanied by a reduction in aspartate aminotransferase level in one patient. However, Weimar et al. (1977) failed to show any effect on serum viral markers in four similar patients following a 2-week course of treatment with fibroblast interferon.

Scullard et al. (1979) treated eight patients with chronic hepatitis B

(seven with chronic active hepatitis and one with chronic persistent hepatitis) with daily intramuscular injections of human leucocyte interferon for periods of 5 to 8 weeks and in one case for 5 months. In one patient there was a marked fall in virus-associated DNA polymerase activity and in the number of DNA-containing viral particles during each of two courses of interferon. Hepatitis B *e* antigen also disappeared, the aspartate aminotransferase levels fell and liver histology improved. In the four other patients with detectable DNA polymerase activity there was an early fall but this was transient, and in one of these patients there was a continuing rise in DNA polymerase activity despite treatment. The *e* antigen became undetectable in two out of three patients in whom it was present. Hepatitis B surface antigen titres were mostly unaffected by treatment. A marked decrease in T lymphocyte-mediated cytotoxicity towards hepatitis B surface antigen-coated target cells was also demonstrated, raising the possibility that the antiviral effect of interferon was offset by its effect on host immune responses to the virus. Interferon has been shown to have a wide range of actions on both cell-mediated and humoral mechanisms, generally of a depressant nature, and the marked decrease in T lymphocyte-mediated cytotoxicity to surface antigen-coated target cells shown in the patients treated by Scullard *et al.* (1979) might reflect a general immunosuppressant effect. This could result in impaired elimination of the virus despite reduced replication.

Merigan and Robinson (1978) treated seven patients with chronic hepatitis B infection with human leucocyte interferon and there was a reduction in the number of complete virus particles in all the patients while on treatment for a month or more. In two out of the seven patients the effect of interferon was sustained after treatment was stopped, resulting in permanent reduction of virus particles, *e* antigen and surface antigen below the level of detectability, and disappearance of the surface antigen and core antigen from liver biopsy. In the third patient there was a permanent reduction in the number of virus particles and *e* antigen, together with a partial reduction in the titre of surface antigen and disappearance of the core antigen from liver biopsy. In another four patients the effect of interferon was reversible with partial reduction in the number of virus particles during treatment only and no change in the titre of the surface antigen.

There seems to be a variable host response to treatment of chronic hepatitis B virus infection with human leucocyte interferon. Persistently infected female patients, particularly before the menopause, appear to

respond better to the antiviral effect of interferon as noted in the studies reported by Merigan and Robinson (1978) and Scullard *et al.* (1979). It is possible that better selection of patients, determination of the optimal dose of interferon for treatment and maintenance therapy and/or combination with an antiviral drug such as adenine arabinoside (see below, and Zuckerman *et al.*, 1978) will achieve a more complete and durable effect on replication of hepatitis B virus.

ANTIVIRAL DRUGS

Most substances with antiviral activity have been identified by empirical testing of a large number of chemical compounds in an attempt to inhibit selectively the synthesis of viral components at concentrations that will not destroy the normal metabolic activities of the host cell. There are, however, several biological and biochemical processes that are essential for viral replication and which are not paramount to survival of the cell. These processes may be more appropriate targets for selective attack by antiviral drugs and may be listed as follows:

1. Attachment of the virion to specific cell receptors
2. Transcription of early viral mRNA by the transcriptase of the virion
3. Translation of early proteins from viral mRNA
4. Replication of viral nucleic acid by virus-coded polymerases
5. Post-translational cleavage of proteins and assembly of virions
6. Regulation of viral gene expression.

The following is a brief summary of information on the prospects of antiviral chemotherapy of viral hepatitis. Review of the literature reveals a remarkable variation in the interpretation of results of various studies, and conflicting observations abound.

Ribavirin (virazole)
Ribavirin is 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, a synthetic nucleoside analogue of guanosine with a molecular weight 244·21, soluble in water with a broad spectrum of antiviral activity. The precise mode of action of ribavirin is not fully understood at present but *in vitro* studies have shown a reproducible, though modest, inhibition of a wide range of DNA and RNA viruses. X-ray analysis of the crystal has shown that the molecular configuration of ribavirin resembles that of guanosine. Ribavirin-5'-phosphate is a potent competitive inhibitor of IMP dehydrogenase isolated from *Escherichia coli* and Ehrlich ascites tumour cells, which

suggests that the antiviral activity of ribavirin may be mediated by in-hibition of GMP biosynthesis at the stage of conversion of IMP to XMP. This is consistent with the reversal of the antiviral action of ribavirin by guanosine and xanthosine.

Ribavirin has been given orally to patients in a dose of 800 mg daily for a month without evidence of toxicity. This drug has been licensed for use in patients in many countries and it is under extensive evaluation for the treatment of a number of viral infections.

Several clinical studies in South America with ribavirin have yielded encouraging results in acute hepatitis B as well as in persistent carriers of the surface antigen (Galvao and Castro, 1974; Vilelea et al., 1974; Zuniga et al., 1974; Huggins and Pereira, 1977). However, no significant changes in serum hepatitis B surface antigen were noted in two adult chimpanzee carriers by Denes et al. (1976) nor in other markers of infection with hepatitis B virus in four chimpanzee carriers treated by Zuckerman et al. (1978).

Treatment of hepatitis B carriers with adenine arabinoside (ara-A, vidarabine)
Arabinosyl nucleosides were first isolated in 1950 from a Caribbean sponge, *Crypotethia crypta*, and later produced by fermentation of *Strepto-myces antibioticus* as potential anti-tumour agents. Adenine arabinoside or ara-A acts as an analogue of the deoxyribonucleoside of adenine, and its significant advantage over the counterpart pyrimidine analogues is its virtual lack of acute toxicity.

Ara-A was shown in 1963 to have significant antiviral activity *in vitro* against herpes simplex virus and vaccinia and subsequently other DNA viruses including type 2 strains of herpesvirus, herpes saimiri, herpes B, pseudorabies, varicella-zoster, cytomegalovirus and myxoma. It has proved especially effective against vaccinial and herpes virus keratitis, herpes ence-phalitis and skin lesions, both experimentally and in clinical investigations.
Metabolism. Ara-A is rapidly deaminated *in vivo* and converted to hypo-xanthine arabinoside (Ara-Hx). Ara-Hx *in vitro* possesses about one-tenth of the antiviral activity of ara-A, although *in vivo* its antiviral efficacy is similar, thus providing a distinct advantage over ara-C and iodo-deoxyuridine which have inactive metabolites. There is also experimental evidence that ara-Hx is re-aminated *in vivo* to form ara-A, leading to an intracellular balance of prolonged antiviral activity.
Mode of action. The precise mode of action of ara-A is uncertain. There is evidence that the portion of ara-A which is converted to adenine ribo-

nucleotides or deaminated to ara-Hx is converted to ara-AMP and to ara-ATP. This leads, in turn, to inhibition of viral DNA polymerase, inhibition of viral ribonucleotide reductase, or inhibition of other virus-specific enzymes involved in viral DNA synthesis. The mechanism of action of ara-Hx is not known.

The drug and its metabolites concentrate intracellularly particularly in red cells, the liver, the kidney and spleen.

Method of administration. The maximum solubility of ara-A in water is 0.05%; and 0.18% in 0.1 M phosphate buffer at $37°C$. This low solubility is associated with moderate or severe irritation when injected intramuscularly. Intravenous infusion is preferred.

Toxicity. Rhesus monkeys tolerate 15 mg kg^{-1} day^{-1} solution of ara-A injected intravenously for 28 days without evidence of toxicity. When given 25 mg kg^{-1} day^{-1} they develop signs of neurotoxicity. Teratogenic effects have not been observed. New World monkeys tolerate much higher doses without evidence of toxicity.

Side effects. During a 2-year trial in 42 patients with severe herpes infection, nausea and vomiting occurred in 76% of the group treated with 20 mg kg^{-1} day^{-1} and in 20% of those receiving 10 mg kg^{-1} day^{-1}. Weakness was noted in 40% of the former group and in 30% of the latter.

In another study involving 143 patients treated for 1 day to 1 month, there was no evidence of hepatotoxicity, nephrotoxicity or haematological changes. Adverse reactions were recorded in 30 patients; and in all but four these were mild to moderate gastro-intestinal symptoms, principally nausea and/or vomiting. Metoclopramide or prochlorperazime are usually effective in controlling nausea.

Two patients with chronic active hepatitis B were treated by Pollard *et al.* (1978) with two courses of adenine arabinoside in a dose of 15 mg kg^{-1} day^{-1} for 9–14 days. In one patient there was a rapid decrease in the level of DNA polymerase activity during both courses of treatment, separated by an interval of 7 weeks, and an increase in DNA polymerase after stopping treatment. There was no significant change in the complement-fixation titre of the surface antigen. In the second patient there was a decrease in DNA polymerase to undetectable levels by the 8th day of treatment and it had not returned to pretreatment level by the 22nd day when the second course of treatment was begun. The second course resulted in reduction of polymerase activity to undetectable levels which had persisted for 12 months. The titre of surface antigen decreased from $1 : 256$ to $< 1 : 8$. Side effects included gastro-intestinal symptoms with

substantial but transient weight loss. In another study, Chadwick *et al.* (1978) reported that ara-A inhibited replication of hepatitis B virus in patients with chronic liver disease, but virus particles did not disappear from the blood, presumably because the particles were cleared more slowly. The patients were treated for only 10 days and treatment resulted in an immediate loss of DNA polymerase activity. In three of the four patients DNA polymerase returned when treatment was stopped, but in the fourth patient, who remained negative for DNA polymerase, the titre of the surface antigen fell. Similar, but temporary effects were noted by Zuckerman *et al.* (1978) in chronically infected chimpanzees treated with ara-A. Merigan and Robinson (1978) treated with ara-A patients with persistent hepatitis B infection, and in all these patients there was a reduction in the number of circulating virus particles while the drug was administered. In two patients this effect was durable; virus particles and *e* antigen could no longer be detected after completion of the course of treatment, and there was a partial reduction of surface antigen from liver biopsy. In another two patients, there was a reduction in the number of virus particles only during treatment but no change in the titre of the surface antigen. It remains to be determined whether continued treatment with this drug alone, or in combination with interferon, will have a more sustained effect on replication of hepatitis B virus.

Other drugs

The flavonoid, (+)-cyanidanol-3, has been shown to decrease the hepatotoxicity of ethanol and other compounds in laboratory animals, and to be a powerful free-radical scavenger. It has also been reported that this drug decreased the level of serum bilirubin in patients with acute viral hepatitis. Blum *et al.* (1977) carried out a double blind trial with this drug and placebo tablets in 100 patients suffering from acute viral hepatitis. The results showed that the drug led to a more rapid lowering of serum bilirubin during treatment, with the difference attaining statistical significance during the 3rd and 4th week of illness. Symptoms such as nausea, anorexia, pruritus and possibly abdominal discomfort were also relieved. Hepatitis B surface antigen had disappeared by the end of 4 weeks of treatment in a greater proportion of the drug-treated than the placebo group of patients. Further reports on the use of (+)-cyanidanol-3 in viral hepatitis have not yet been published, but from the report by Blum *et al.* (1977) the beneficial effects of this drug appear to be marginal and mainly subjective.

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Subject Index

References to figures and tables are in *italics*.