

HEPADNAVIRUSES, THEIR INFECTIONS AND HEPATOCELLULAR CARCINOMA

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ABSTRACT

Ten years ago hepatitis B virus (HBV) was thought to be a unique virus, not included in any known family of viruses. Following the discovery of a number of HBV-like viruses that infect birds and mammals, the existence of a new family known as hepadnaviridae has been confirmed. Hepadnaviruses are small hepatotropic viruses that have a characteristic partially double stranded genome, exhibit a narrow host range and replicate by reverse transcription¹. The family currently comprises six viruses of which human hepatitis B virus is the prototype member. Other members include woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV), tree squirrel hepatitis virus (TSHV), Peking duck hepatitis B virus (DHBV) and heron hepatitis B virus (HHBV)²⁻⁶. Candidate members of the family include kangaroo hepatitis virus (KHV) and stink snake hepatitis virus (SSHV). In humans, infection with HBV is associated with a wide spectrum of clinical conditions (Table I) including acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). Infection with HBV is endemic throughout much of the world and the virus is maintained by the enormous reservoir of over 300 million chronic carriers (Table 1). For almost 20 years experimental work on hepadnaviruses has been carried out using either natural hosts or cultured cells that were capable to support synthesis of a few viral gene products but unable to execute a complete cycle of virus replication. In this article, we have attempted to summarize the efforts made towards understanding the biology of hepadnaviruses, the nature of their infections and their association with primary liver cancer (JPMA 40 :300, 1990).

HISTORICAL PERSPECTIVE

Jaundice has been known since antiquity and has been recorded in Egyptian and Indian Ayurvedic texts and the Babylonian Talmud. Many indigenous medical systems contain remedies for the treatment of jaundice some of which was probably due to HBV infection. The first cases of serum hepatitis (hepatitis B) to be recorded apparently occurred following administration of small pox vaccine, containing glycerinated human lymph, to shipyard workers in Bremen in 1883. Of 1289 vaccinated employees, 191 developed jaundice after intervals ranging from several weeks to 8 months. Successful experimental transmission to human volunteers finally established the viral aetiology of hepatitis⁷ and later it was confirmed that there were two distinct epidemiological, clinical and serological types of viral hepatitis⁸. However, the modern understanding of hepatitis B virology began with the discovery of Australia antigen in the serum of an Australian aborigine in 1965⁹ and its association with hepatitis two years later¹⁰. The realization that this antigen was an envelope protein of the causative agent of hepatitis B rapidly led to the development of diagnostic assays that could be applied to seroepidemiological studies of human populations. With this simple approach, it became possible to demonstrate that HBV was a major human pathogen causing considerable morbidity and mortality around the world from conditions such as acute and chronic infections, cirrhosis and primary hepatocellular carcinoma¹¹. It was also shown that the Australia antigen, now known as hepatitis B surface antigen (HBsAg) (Table II),

would form the basis for one of the world's most successful subviral vaccines and the first genetically engineered viral vaccine. Hepatitis antigen was initially detected in the blood by the relatively insensitive gel diffusion technique in 1967-68¹². Techniques reported in 1970 include complement fixation¹³ and counter immuno-electrophoresis¹⁴ which showed 5 to 10-fold greater sensitivity than gel diffusion. Reverse passive haemagglutination revealed approximately 100-fold higher sensitivity than gel diffusion. By 1972 radioimmunoassay (RIA) technique was adapted to the more convenient solid phase systems¹⁵. Currently both RIA and enzyme immunoassay (ELISA) which show almost equivalent sensitivity of detection of HBV markers¹⁶ are used extensively for both diagnostic and epidemiological investigations. Development of a ³²p-labelled HBV DNA probe permitted the detection of viral DNA in the liver cells of some patients with chronic hepatitis B in 1981¹⁷ by Southern Blot technique. HBV DNA was also detected in serum by a dot blot hybridization technique in 1983¹⁸. It is beyond the scope of this article to discuss the molecular biological details of structure and life cycle of hepadnaviruses that have been elucidated over the past two decades. However, a brief summary of the replication cycle of hepatitis B virus that has been derived from studies of samples collected from human and animal tissues, is given under biological aspects of hepadnavirus infection.

HEPADNAVIRUS PARTICLES

The general structure of all other hepadnaviruses is similar to that of HBV. The complete infectious hepatitis B virion (Figure 1)

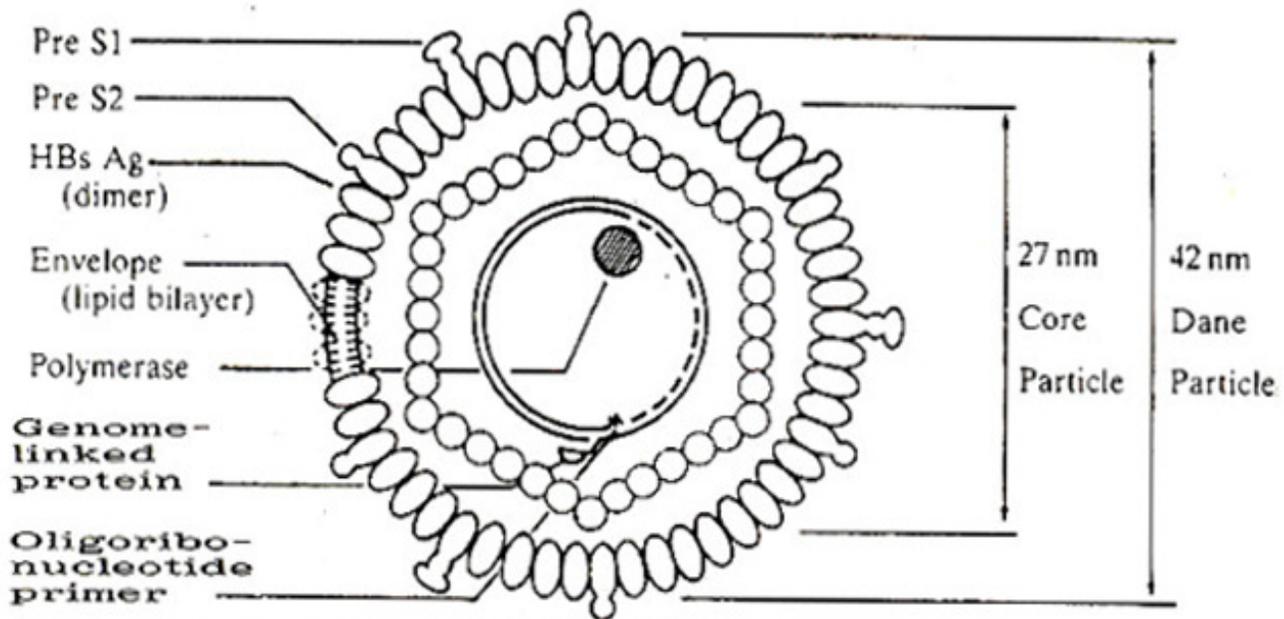


Figure 1. Structure of Hepatitis B virion. The 42nm particle consists of an outer envelope of HBsAgs surrounding a nucleocapsid core of 27nm. The core contains a partially double stranded viral genome, DNA polymerase/reverse transcripts, a genome-linked protein and an oligoribonucleotide primer. The oligoribonucleotide primer is linked to the 5' end of the incomplete DNA plus strand. The genome-linked protein is covalently attached to the 5' end of the DNA minus strand which also carries an approximately 5-10 nucleotide terminal redundancy.

is a spherical, enveloped particle with a diameter of 42nm. These morphological criteria conform with

the description of the Dane ,particle visualized by electron microscopy¹⁹. HBsAg is the main protein component of the lipid-containing envelope. HBsAg exists as a structural element of the infectibus virus and as spherical and tubular, noninfectious elements in the blood of infected individuals. The chemical composition of HBsAg can be inferred from the buoyant density of the 22nm spherical particles (1.2 g/cm³ in CsCl) which suggests a 30%lipid content. HBsAg is detected in the cytoplasm and cell membranes of infected hepatocytes by immunofluorescence studies. The nucleocapsid with a diameter of 27nm consists of the viral genome and HBcAg.

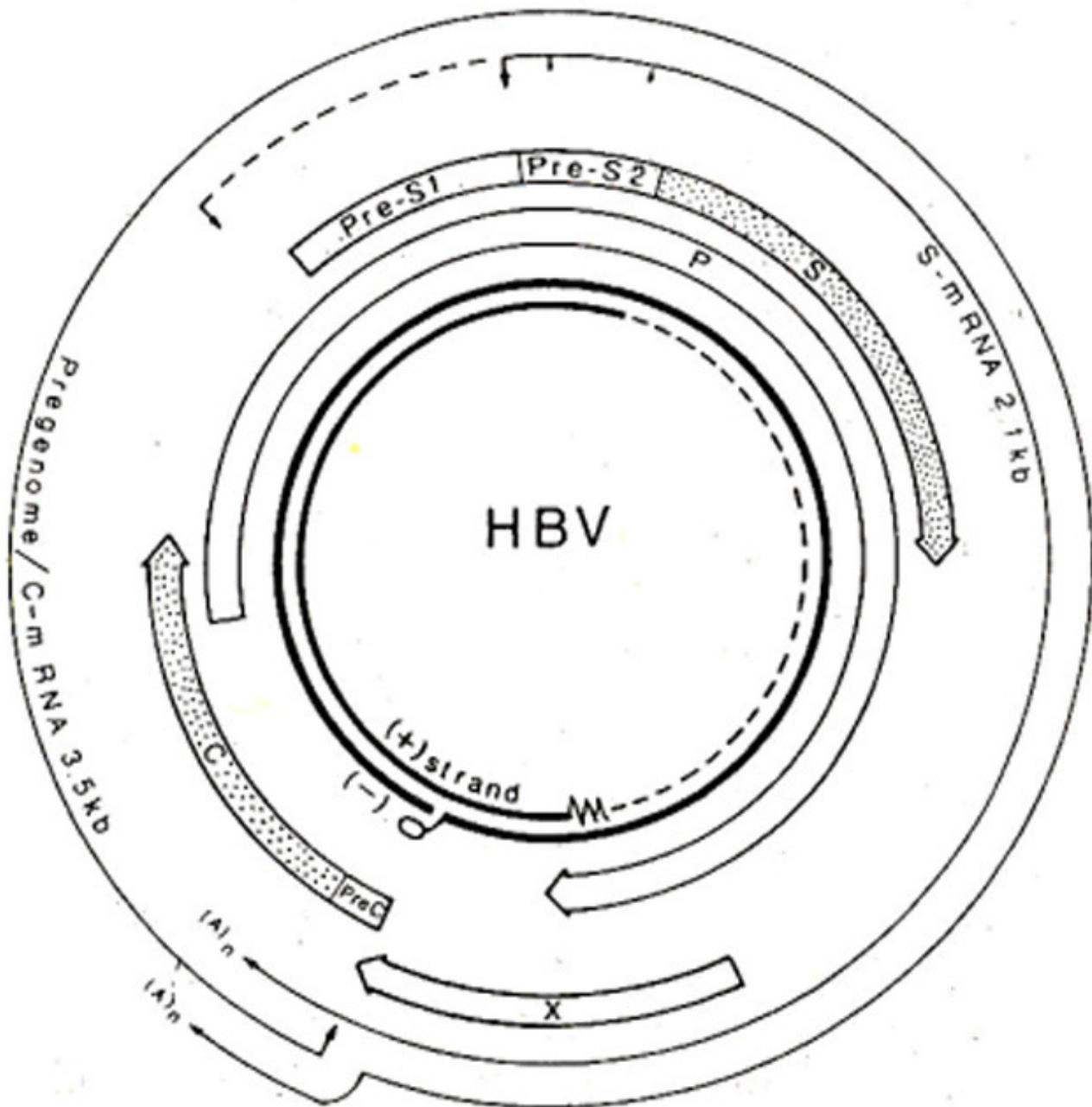


Figure 2. Genetic organisation of hepatitis B virus genome. Centrally located solid lines indicated two strands of the genome. Gap in the plus strand is represented by dotted line. The large arrows immediately surrounding the genome indicate four major open reading frames, S, C, P, and X. The arrows around the outside of the figure indicate the positions of the major families of HBV transcripts.

The genome (Figure 2) is a partially double stranded DNA molecule about 3200 basepairs with a long (minus) and a short (plus) strand²⁰. This genome has an unusual organization and there are similarities between hepadnaviruses and retroviruses²¹. In fact, the hepadna- and retroviruses utilize a unique mechanism of genome replication involving reverse transcription of an RNA intermediate. The long

DNA strand contains the transcription units named as open reading frames (ORF) S, C, P, and X. The ORF S codes for the surface, major surface protein as well as for the large and the middle surface proteins which are expressed from the preS₁ and preS₂ regions respectively. The pre-S₁ protein is involved in attachment of the virus to a hepatocyte receptor²². ORF C codes for the major core protein p²². Expression of core and prescore sequences result in a larger protein which is processed at both ends (Figure 3)

BIOSYNTHESIS OF HBcAg & HBeAg

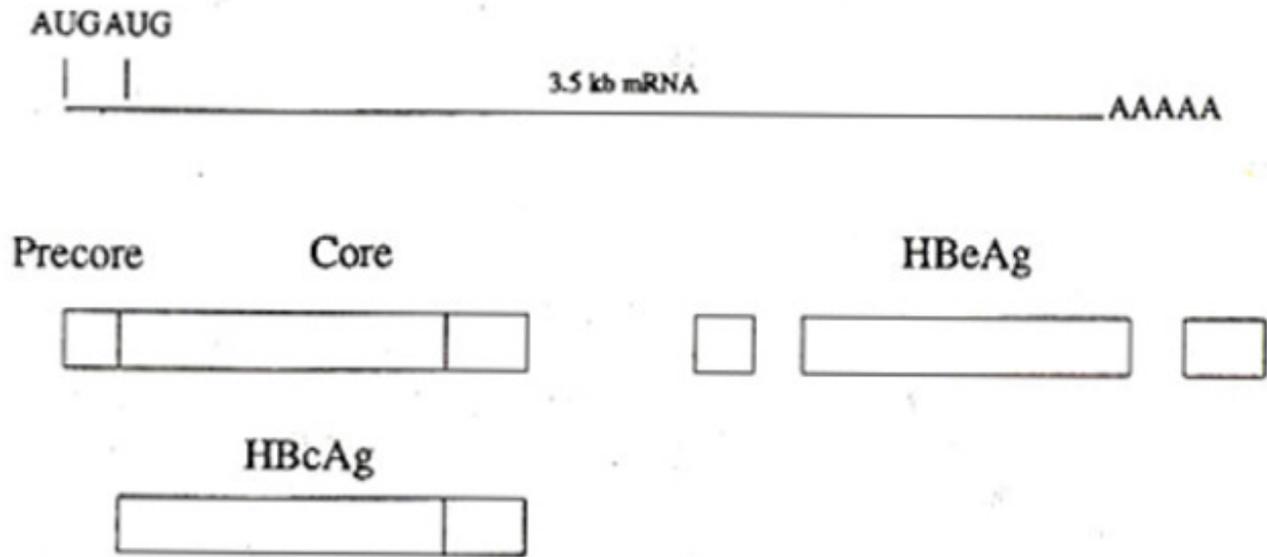


Figure 3. Biosynthesis of HBcAg and HBeAg. Inclusion of the pre-C region leads to the production of a large pre-core polypeptide of 25 kd which is processed at both termini to yield a much smaller HBeAg (17 kd). Origin of the protease activity involved in this cleavage is still obscure. Exclusion of the pre-C region leads to translation of HBcAg (21 kd) without further processing. HBcAg has a propensity for the viral nucleic acid and forms the nucleocapsid by encapsidation of the pregenome

to produce a much smaller secretory hepatitis Be antigen (HBcAg)²³. The P region encodes the viral DNA polymerase which has reverse transcripts activity. The ORF X codes for a protein which functions as transcriptional activator²⁴.

BIOLOGICAL ASPECTS OF HEPADNAVIRUS INFECTION

The biology of hepadnavirus infection involves at least three major aspects, i.e. viral entry into the host cell, viral gene expression and replication, and viral integration into the host DNA.

VIRAL ENTRY INTO THE LIVER CELLS

Hepadnaviruses have a propensity for liver cells. Attachment of virus to the liver cell and its subsequent entry is believed to be mediated through a specific hepatocyte receptor that recognizes the pre-S₁ protein of the virus²⁵. Following entry into the cell, uncoating takes place and the partially double stranded genome is transported to the nucleus where upon repair it is converted into a covalently closed circular DNA (cccDNA) molecule. The cccDNA can either persist in the cell in a latent state, or it can undergo active expression and replication (Figure 4)

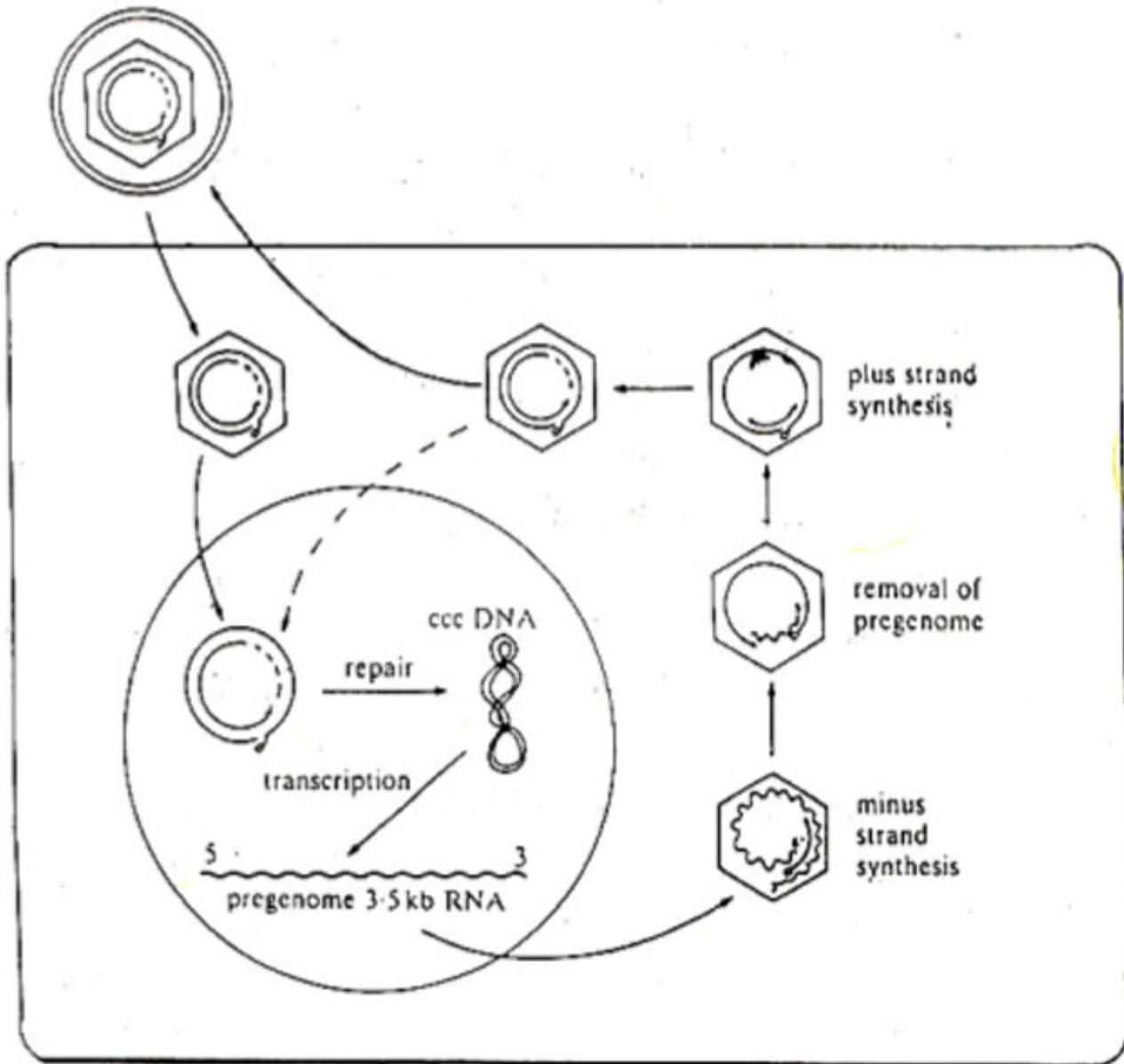


Figure 4 Schematic representation of hepadnavirus replication. Repair of the partially double stranded genome takes place inside the nucleus of infected hepatocyte. The pregenomic mRNA of 3.5 kb is transcribed from the supercoiled version of the genome and subsequently transported to cytoplasm. Further events including encapsidation of the pregenome, reverse transcription, and maturation occur in the cytoplasm.

or it can become integrated into host chromosomes (see hepadnavirus and hepatocellular carcinoma).

VIRAL EXPRESSION AND REPLICATION

The cccDNA serves as template for transcription which commences under the host encoded RNA polymerase II for each transcript. All hepadnavirus transcripts are unspliced, have a positive polarity and terminate at the ubiquitous polyadenylation signal located in the core region of the genome²⁶. Two classes of viral mRNAs are synthesized, i.e., genomic 3.5kb mRNA species containing the full complement of the genetic information and subgenomic mRNA species (2.4, 2.1 and 0.6 kb). The

genomic RNA species consists of subspecies with heterologous 5'terminal depending as to whether transcription begins in the pre-C or C region²⁶. The smaller 3.5kb transcript serves as a template for viral DNA synthesis and the C and P gene products. The larger 3.5 kb transcript serves as mRNA for the prescore/core protein the precursor of hepatitis Be antigen (HBeAg)²⁷. The major subgenomic species 2.1kb transcript serves as a template for expression of the middle (pre-S2) and major (S) surface proteins²⁸. Of the minor subgenomic transcripts 2.4 kb acts as mRNA for the large (pre-S1) protein and 0.65kb acts as mRNA for hepatitis B x antigen (HBxAg)²⁶.

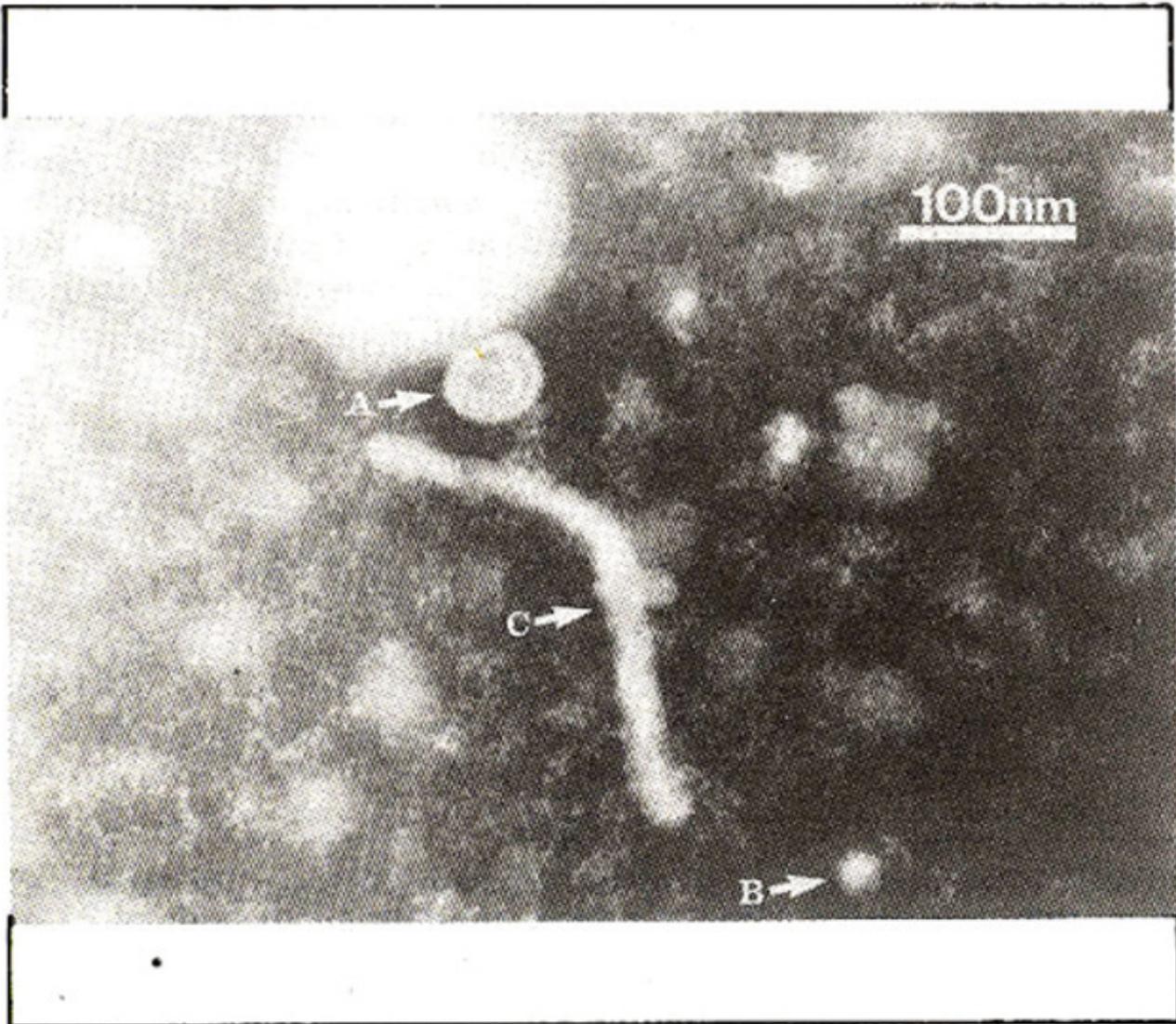


Figure 5. Electron micrograph of HBV and HBsAg particles. The particles were pelleted by ultracentrifugation from harvest of human hepatoma cells, HepG2, transfected with cloned HBV DNA and subsequently immunoprecipitated. The pellet was applied directly to copper grids, stained with phosphotungstic acid and photographed in a Jeol CX 100 electron microscope. The electron micrograph shows A: 42nm complete virion, B: 22nm spherical HBsAg particle and C: filamentous HBsAg particle.

The replicative cycle (Figure 5) of hepadnaviruses is summarized as follows: Virus infects a

hepatocyte, where the relaxed circular genome (Figure 2) is converted to a covalently closed circular DNA (cccDNA). The cccDNA serves as template for synthesis of viral pregenomic RNA in productively infected cells. Later in the infection the pregenomic RNA is transported from the nucleus to the cytoplasm where it is packaged into immature viral cores. Reverse transcription of the pregenome is then primed by the genome-linked protein within the direct repeat 1 (DR1) under the viral encoded reverse transcriptase resulting in the synthesis of minus DNA strand. Following minus strand elongation to the end of the pregenome, plus strand synthesis initiates just downstream of the direct repeat 2 (DR2) utilizing an oh goribonucleotide primer (Figure 1) derived from the 5' end of the pregenome via a translocation event. The short terminal redundancy of the, minus strand facilitates the template switch of the plus strand from the 5' end to the 3' end of the minus strand. The plus strand remains incomplete by 15-50% due probably to the relative depletion of nucleotides because of rapid encapsidation thus leaving a characteristic gap in the hepadnavirus genome (Figure 2). Finally the nucleocapsids are packaged into viral envelopes and somehow secreted from the infected cells²⁸.

CLINICAL ASPECTS OF HEPADNAVIRUS INFECTION

The incubation period of acute hepatitis B ranges from 50 to 150 days, shorter after massive exposure. It may be prolonged beyond 6 months after administration of specific immunoglobulin. The onset is insidious. After a few days of increasing anorexia, accompanied by nausea and vomiting and elevation of temperature to 38-39°C, jaundice sets in, sometimes accompanied by hepatomegaly and abdominal pain, following which the temperature returns to normal. These symptoms indicate the host immune response to virus. The urine becomes dark due to excretion of urobilinogen and bilirubin, and faeces become pale due to lack of bilirubin excretion by damaged hepatocytes. Generally the majority of cases are mild and anicteric. Characteristic biochemical findings include raised serum levels of alanine amino transferase (ALT) (formerly known as serum glutamic pyruvic transaminase) and aspartate aminotransferase (AST) (formerly serum glutamic oxaloacetic transaminase). Total serum bilirubin levels in jaundiced patients usually exceed 2.5 mg/dl. Clinical recovery occurs after several weeks or months but some individuals eventually develop persistent or chronic hepatitis with progression to cirrhosis and or hepatocellular carcinoma. Occasional patients may develop fulminant hepatitis, with abrupt onset of fever, jaundice, drowsiness progressing to stupor and coma, followed by death within 2 or 3 days with acute yellow atrophy of the liver. Similar symptoms may be seen in extrahepatic obstruction, cholecystitis, liver cancer, drug-induced hepatitis, hepatitis caused by other viruses such as hepatitis A virus, hepatitis C virus, delta agent, hepatitis non-A, non-B virus, cytomegalovirus and Epstein Barr virus. A definite aetiological diagnosis must be based on laboratory tests. HBV appears in blood before HBsAg can be demonstrated. In about 90% of patients HBsAg disappears from the blood in about 3-4 months. In most cases the liver function tests return to normal within this period, while about 5% have raised aminase levels for up to a year. In such patients histological signs of chronic persistent hepatitis are often found. If HBsAg persists for more than six months antigenaemia is considered to be chronic and the patient becomes a chronic carrier. However most chronic carriers have no history of a symptomatic liver infection. HBeAg can usually be demonstrated shortly after the appearance of HBsAg and is replaced by anti-HBe before HBsAg disappears. Anti-HBs usually appears when HBsAg can no longer be detected, in some cases week or months after disappearance of HBsAg (serological window)²⁹. While the presence of anti HBs indicates immunity, the presence of HBsAg is highly indicative of active HBV production. Chronic carriers are usually tested for HBeAg/anti-HBe, as the presence of HBeAg is classically related with active HBV replication and the finding of anti-HBe generally indicates low infectivity and disappearance of virus.

HEPADNAVIRUS INFECTION AND HEPATOCELLULAR CARCINOMA

The epidemiological association between HBV and hepatocellular carcinoma (HCC) has been firmly established³⁰. Longstanding chronic HBV infection and the development of chronic liver disease ultimately but not inevitably into cirrhosis are associated with a high risk for the development of

primary hepatocellular carcinoma. HBV DNA was first shown to be integrated into a hepatoma cell line³³ and then it was demonstrated in human tumour tissue³². Further studies have revealed the presence of integrated HBV DNA in patients with chronic hepatitis³³. There is some controversy as to whether integrated HBV DNA is more important in the pathogenesis of HCC or cirrhosis. However, the involvement of HBV in the pathogenesis of HCC has been established in a number of ways. There is a high incidence of HCC in areas with a high incidence of chronic HBV infection. There is an incidence of serum HBV markers in patients with HCC³⁴. HBsAg was demonstrated in the tumour and non-tumour tissue of patients with HCC. Some human hepatoma cell lines secrete HBsAg and hepatoma develops when these cells are injected into nude (athymic) mice. Of the other hepadnaviruses WCHV is closest to the human virus as it causes persistent infection and HCC in woodchucks³⁵. Primary hepatocellular carcinoma is one of the ten most common forms of cancers in the world, and is especially prevalent in developing countries. It is uncommon in Europe or the USA but is one of the most frequently observed fatal malignancies in Asia and Africa, particularly in young men. HCC has an average latent period of 20 to 40 years. For reasons that are still unclear, men are about 4 to 6 times more likely than women to develop HCC. HCC claims about one million lives around the world each year. Current estimates based on epidemiological studies indicate that 25% of those who are infected at birth and develop chronic hepatitis will eventually die of liver cancer and/or cirrhosis (Table I).

TABLE I. Spectrum of Hepatitis B virus infection.

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1. More than 2 billion people have been infected globally.
 2. More than 300 million are now chronic carriers.
 3. World pool of HBV carriers:
 - Asia 78%
 - Rest of the world 22%
 4. 1-2 million deaths per year are directly related to HBV infection.
 5. 5-10% of adults and 70-90% of infants infected with HBV remain chronically infected.
 6. 25% of chronic carriers die of HCC or cirrhosis.
 7. HBV causes upto 80% of the world's primary liver cancer.
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A prospective general population study started in Taiwan in 1974 provided convincing evidence that links HCC to HBV. This large cohort study included 22,707 Chinese male civil servants. Preliminary data showed that subjects who were chronic HBV carriers had an approximately 200 fold increased risk of developing HCC compared with HBsAg-negative subjects³⁶. Symptoms of liver cancer include weight loss, abdominal pain which tends to intensify after eating, jaundice, muscle wasting and ascites. There is no adequate treatment available for liver cancer at present. However, hepatitis B vaccine by preventing HBV infection and its sequelae, eliminates a major cause of liver cancer.

CONTROL OF HBV INFECTION

HBV is an important human pathogen and is responsible for considerable morbidity and mortality around the world. Therefore there is an urgent need for control of the virus. At present, there is no specific therapy for acute HBV infection. Hospitalization is not needed in mild acute cases. Treatment with interferon and antiviral agents may possibly have a favourable effect in some patients with chronic HBV infection. Measures are therefore directed mainly towards prevention of HBV infection. The most

effective means of protection against HBV infection is the hepatitis B vaccine. Following demonstration by Krugman et al in 1970³⁷ that boiled HBsAg-laden plasma induced resistance to challenge by live HBV, it was found by 1980 that purified preparations of HBsAg derived from plasma of HBV carriers induced anti-HBs and resistance to natural HBV infection in upto 85-95% vaccines^{38,39}. The plasma derived purified HBsAg particles were used as sub-unit HBV vaccine in the earlier trials. The preparation involved precipitation by ammonium sulfate from plasma of chronic HBs Ag carriers, isopycnic banding on sodium bromide, sucrose gradient-rate zonal centrifugal, and enzymatic digestion with pepsin³⁸. The purified HBsAg were further treated with formalin 1:4000 for 72 hours at 39°C to kill any possible residual live HBV or concomitant viruses, such as human immunodeficiency virus (HIV). Subsequent trials were conducted using HBsAg particle inactivated by pepsin, urea and formalin and formulated in 1 ml doses containing 40ug antigen in alum adjuvant³⁹. This preparation was administered as 0.5 ml doses containing 20 ug HBsAg by intramuscular route at 0, 1 and 6 months intervals. Immunogenic polypeptides within HBsAg are in two forms, a polypeptide of 25 kd (p25) and its glycosylated form of 30 kd (gp30)⁴⁰. The p25/gp30 complex has been prepared by extraction from native HBsAg by treatment with Triton X-100 and aggregated into micelles of about 200nm diameter for use as vaccine⁴¹. Genetically engineered vaccine has been prepared by using recombinant DNA technology. The region of HBV genome coding for the 25 antigenic determinant has been cloned and expressed in yeast *Saccharomyces cerevisiae*, the resulting protein adsorbed on aluminum hydroxide and prepared as 1 ml doses of vaccine containing 10 ug HBsAg strain adw⁴². Seroconversion rates of 93-95% were achieved 3 months or more after commencement of vaccination with either plasma-derived or recombinant vaccines⁴². Hepatitis B vaccine is thus the most effective tool in preventing transmission of HBV infection. When administered properly hepatitis B vaccine induces protection in about 95% of recipients. The full doses as recommended by the manufacturers should be administered intramuscularly at 0, 1 and 6 months. Excellent seroconversion (upto 99%) can be achieved with 3 doses. HB vaccine should not be frozen as this will destroy its potency. The recommended temperature for storage is between + 0 and + 8°C, the same as for DPT vaccine. HB vaccine does not interfere with DPT, OPV, BCG and measles vaccines and can be administered concomitantly. In cases of needlestick injury, or by mucosal exposure to blood or body fluids which may harbour HBV, protection can be achieved in most cases by passive immunization. It is recommended that 0.06 ml/kg hepatitis B human immune globulin (HBIG) be administered preferably within 24 hours of exposure and repeated one month later⁴³. Hyperimmune serum administration should be followed by immunization with plasma-derived or recombinant HB vaccine. As there is high risk of perinatal transmission through exposure to maternal blood and/or faeces during the birth process, HBIG is also recommended for administration on the day of birth to all infants born to HBsAg-positive mothers. The vaccine may then be given at a later stage to provide additional protection⁴⁴.

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