CHAPTER II

REVIEW AND RELATED LITERATURES

Hepatitis B Virus : historical review

An outbreak of parentally transmitted hepatitis was probably first documented in 1883 by Lurman in Bermen, Germany who reported the occurrence of jaundice among personnel of a Bremen factory after revaccination against smallpox [18]. Thousands of persons received vaccine that had been prepared from human lymph. Of 1,289 individuals vaccinated in one day, 191 (15%) developed jaundice 2 to 8 months later and jaundice did not occur among unvaccinated workers.

In the 19th century, MacCallum and Bauer proposed, on the basis of differences in incubation period and mode of transmission, the current nomenclature of hepatitis A for infectious hepatitis and hepatitis B for "homologous serum" hepatitis [19]. At that time, it was known that hepatitis A was transmitted by the fecal-oral routes, had an incubation period of 2 to 6 weeks and was primarily a disease of younger children while hepatitis B was transmitted by percutaneous exposure to blood products, had a range of longer incubation period from 2 to 6 months, and occurred more often in adults. In 1965, Blumberg *et al.* had already discovered an antigen in the serum of an Australian aboriginal which he called "Australian antigen" [20]. Subsequently, Prince (1968) identified an antigen in the serum of patients with post-transfusion hepatitis, an antigen which he called SH antigen [21]. The antigen discovered by Blumberg and Prince were found to be identical and represent the HBsAg. The other principal viral antigens (HBeAg, HBcAg) and their antibodies were identified between 1968 and 1973 [22-23].

The viral etiology of hepatitis B was firmly established by electron microscope in 1970 by Dane *et al.* [24]. In the blood of infected patients, the large complete virus particle with diameter 42 nanometer (nm), referred to as "Dane particles", and the detection of several viral particles including small 22 nm spherical surface antigen particles and tubular forms (length 100 nm, diameter 22 nm) were found.

Virology of Hepatitis B virus

HBV is a partial double stranded, enveloped DNA virus of the *Hepadnaviridae* family, which replicates in the liver and causes hepatic dysfunction and associates with the development of hepatocellular carcinoma [25]. The structure of HBV is shown in Figure 1 [26]. The outer envelope composed of three related proteins: PreS1, PreS2 and HBsAg protein. HBsAg is found on the surface of the virus and produced in excess amount as 22 nm spherical and tubular particles. The inner component of the infectious virion contains the core antigen (HBcAg). Inside the core particle is a single molecule of partially double-stranded DNA and an endogenous DNA-dependent DNA polymerase. A third viral antigen, HBeAg, is a soluble protein closely related to core antigen. The presence of HBeAg in blood is associated with high levels of viral replication.

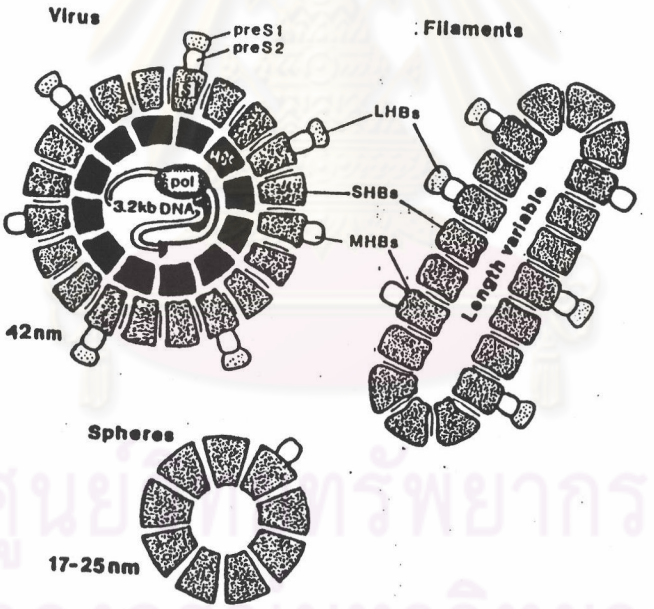

Figure 1 Schematic diagram of hepadnavirus particles. Individual subunits containing SHBs protein only, HBs protein plus PreS2 (MHBs), and HBs protein plus PreS1 and PreS2 (LHBs) are shown in intact virus, among filaments and spheres. The viral particles contain an internal nucleocapsid (HBc), viral genome and polymerase enzyme (pol.) [26]

HBV is the smallest DNA virus known : it has only 3,200 basepairs (bps) in its genome, which is organized in a partly double-stranded, circular pattern (Figure 2) [27]. The minus strand of the DNA is almost a complete circular and contains overlapping genes that encode both structural proteins (surface and core) and replicative proteins (polymerase and X protein). The plus strand of the DNA is shorter and variable in length, 50-100 % of the minus strand length [8].

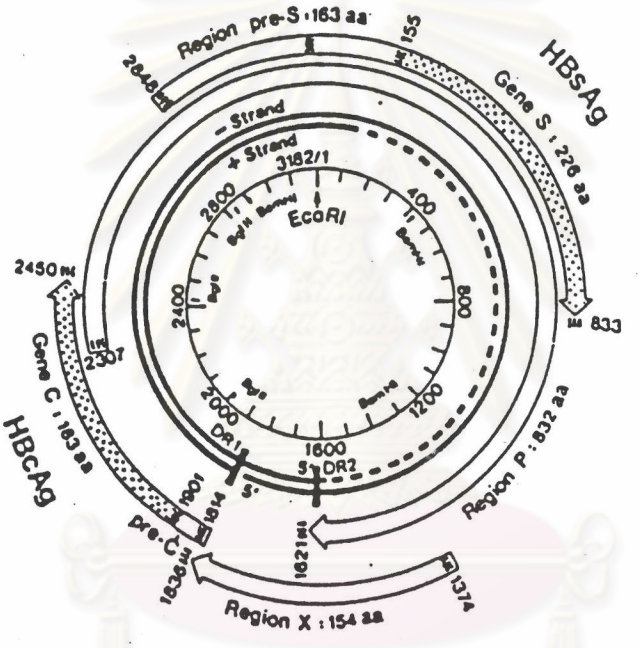

Figure 2 Structure and organization of the HBV genome [27]

The position of the 5'-end of both strands is fixed, while the position at the 3'-end of the plus strand is variable. Despite the nick at position 1820 of the minus strand, the circularity of the genome is maintained by the 224 bps 5' cohesive terminus [28]. At both sites of this cohesive terminus there is an 11 bps direct repeat (DR1 and DR2), that is involved in the initiation of viral DNA synthesis. The sequence of the minus strand starts within DR1, where as that of the plus strand starts closely upstream of DR2 [29].

Four mRNA transcripts of known function have been identified as being involved in HBV transcription and translation [30]. The 3.5 kilobase (kb) transcript is bifunctional : it serves as a template for reverse transcription and as mRNA for DNA polymerase, precore/core (HBcAg) and HbeAg. The 2.4 kb transcript encodes PreS1,PreS2 and HBsAg (large surface protein, LHBsAg) while a 2.1 kb transcript encodes only PreS2 and HBsAg (middle surface protein, MHBsAg). The smallest transcript 0.7 kb encodes X protein.

HBcAg and HBeAg are translated from *C* gene. When transcribed, HBcAg is targeted to the endoplasmic reticulum; here it is cleaved and HBeAg (the precore fragment) is secreted. HBcAg is essential for viral packaging and is an internal part of the nucleocapsid. It is not detectable in serum; however, it can be detected in liver tissue in patient with acute or chronic HBV infection. HBeAg is a soluble protein that can be detected in the serum of patients with high virus titer; it is not essential for viral replication [30].

The surface gene encodes for the virus envelope. The major protein that forms the HBsAg particles is the smallest gene product (SHBsAg). Middle and large surface proteins are found in larger proportions in the intact virus particles.

HBV replication

According to the model proposed by Summers and Mason (1982), the replication of HBV is thought to take place as represented in Figure 3 [31].

After attachment of the virus to the plasma membrane of the host cell, the viral particle is somehow uncoated and the viral core particles enter the cell. After entering the cell, the viral genome is transported to the nucleus. The plus strand is extended and the viral DNA is made fully double-stranded. The terminal protein on the minus strand is removed and the viral DNA is converted into covalently closed circular DNA (cccDNA) [32]. This cccDNA functions as a template for transcription with the generation of genomic (3.5 kb) RNA and subgenomic (2.4 kb, 2.1 kb and 0.7 kb) RNAs coding for viral proteins. Detection of cccDNA, precore/core mRNA and X mRNA are therefore reliable parameters for a successful HBV infection [33].

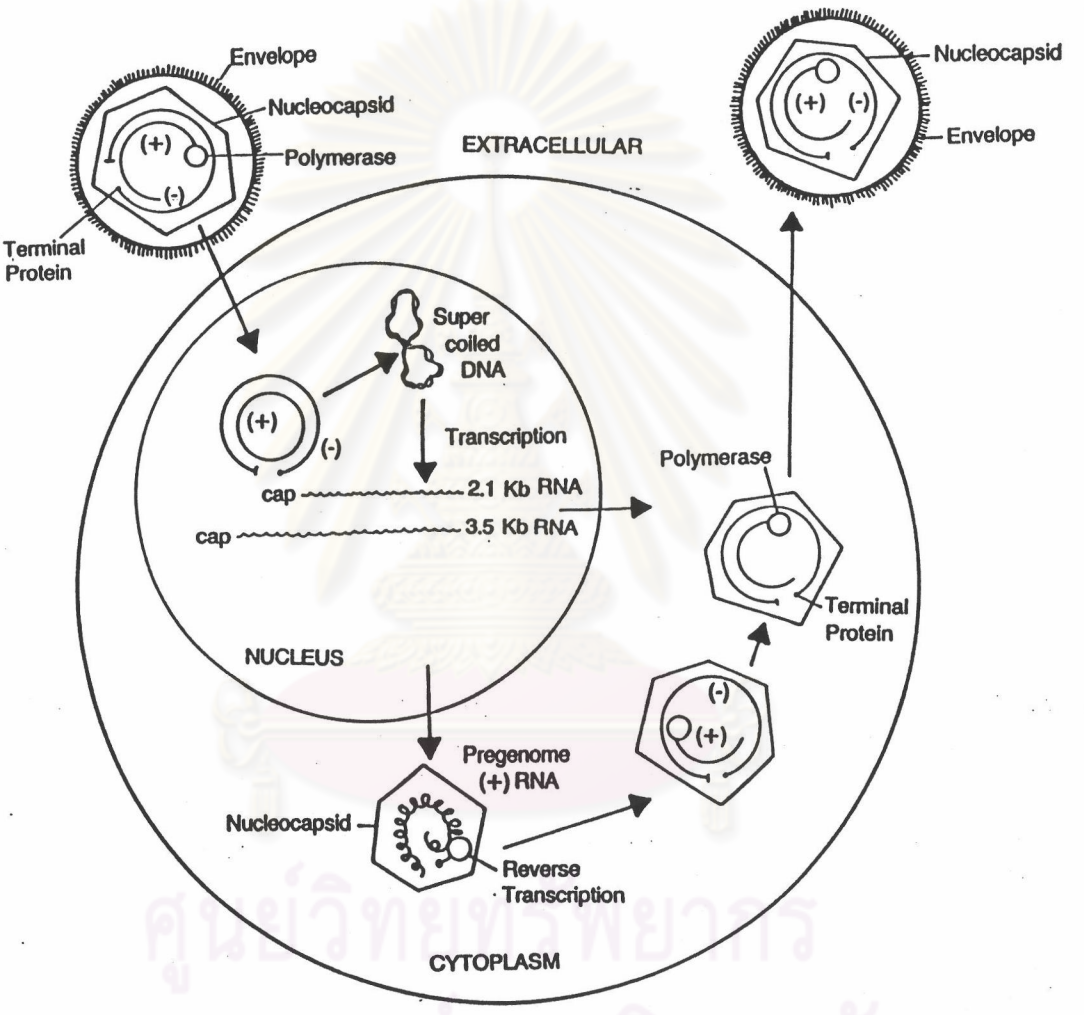

Figure 3 Schematic view of the life cycle of HBV [31]

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After transcription step, these RNAs are translocated from the nucleus to the cytoplasm where they are translated. The pregenomic RNA (full length plus strand RNA) and the terminal protein / reverse transcriptase / RNase H / DNA polymerase polyprotein are packed within HBcAg and form core particles. This encapsidation reaction is highly specific, since neither non-viral RNAs nor subgenomic RNAs are encapsidated [34]. The pregenomic RNA primed by the terminal protein of the *P* gene is then reversed transcribed into minus strand DNA [35] and as the synthesis progresses, the RNA template is simultaneously degraded by RNaseH [32]. After the completion of the minus strand, the viral plus strand DNA is synthesized, utilizing the minus strand DNA as template and probably the short residual oligoribonucleotide fragment of the pregenomic RNA as primer [32] . Finally, the core particles are coated with an envelope consisting of HBsAg and host derived lipids and they are exported from the cell. Completion of the plus strand is not an absolute requirement for coating and release from the cell, as extracellular virions may contain incomplete plus strands and even HBV DNA-RNA hybrids [36] .

Clinical outcome and pathogenesis of HBV infection

Exposure to HBV may result in very different clinical outcomes ranging from asymtomatic hepatitis, acute anicteric or icteric hepatitis, fulminant hepatitis or chronic hepatitis to the development of liver cirrhosis and hepatocellular carcinoma in the long term.

The consequences of the acute HBV infection are highly variable. The incubation period of the virus is anywhere from 6 weeks to 6 months. Newborns generally do not develop any clinical signs or symptoms, and infection produces typically illness in only 5 to 15% of children 1 to 5 year of age and only 33 to 50% of older children and adults are symptomatic [37] .

Chronic HBV infection is defined as the presence of HBsAg in serum for more than 6 months and the absence of anti-HBs and anti-HBc immunoglobulin (IgM). The risk of developing chronic infection varies inversely with age and is highest (up to 90%) in infants infected during the perinatal period [37]. Between 25 and 50% of children

infected between 1 and 5 years of age develop chronic infection, compared to 6 to 10% of acutely infected older children and adults. Chronic carriers are at higher risk for developing cirrhosis of the liver (chronic liver disease) or liver cancer (hepatocellular carcinoma).

Interestingly, the probability of becoming a chronic carrier of HBV is inversely related to the age at infection (Figure 4). Therefore the younger one is at time of infection, the greater the likelihood of becoming a chronic carrier of HBV and thus developing severe liver problems and transmitting the infection to others [38].

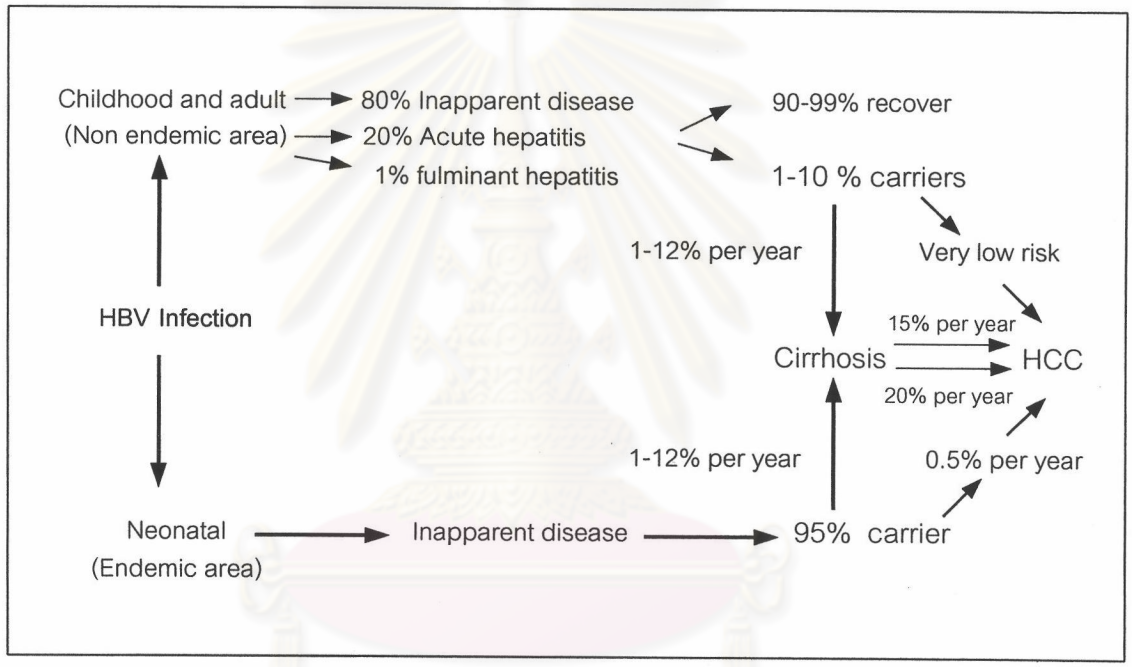

Figure 4 Course of HBV infection after neonatal or adult exposure [38]

The pathogenesis of both acute and viral hepatitis is slowly being unraveled. Thus far, most data show that HBV is not directly cytopathic. Instead, damage to the liver is mediated by the host defence mechanisms against the HBV infection [39]. Traditional symptoms of liver dysfunction cause by HBV infection including the jaundice, nausea (with or without vomiting), fever, weakness, abdominal pain, joint soreness and dark urine.

These symptoms are common to all viral hepatitis infections. In order to obtain a definitive diagnosis it is necessary to do a serological test to confirm the presence of HBsAg . In acute infection, HBsAg and immunoglobulin M (IgM) will be present in the serum (Figure 5). The IgM is anti-HBc immunoglobulin and anti-HBe immunoglobulin. The presence of anti-HBsAg IgG indicates convalescence of the acute infection as this is the neutralizing antibody that serves as the correlate of immunity for HBV. Its presence indicates the end of acute infection and a state of immunity towards HBV. In contrast, HBsAg itself can be detected in the serum of chronic carriers for more than 6 months, often for life and anti-HBs never appears. In addition, the HBeAg is also detectable, while the IgM anti-HBcAg becomes indetectable about 6 to 9 months post infection [16] .

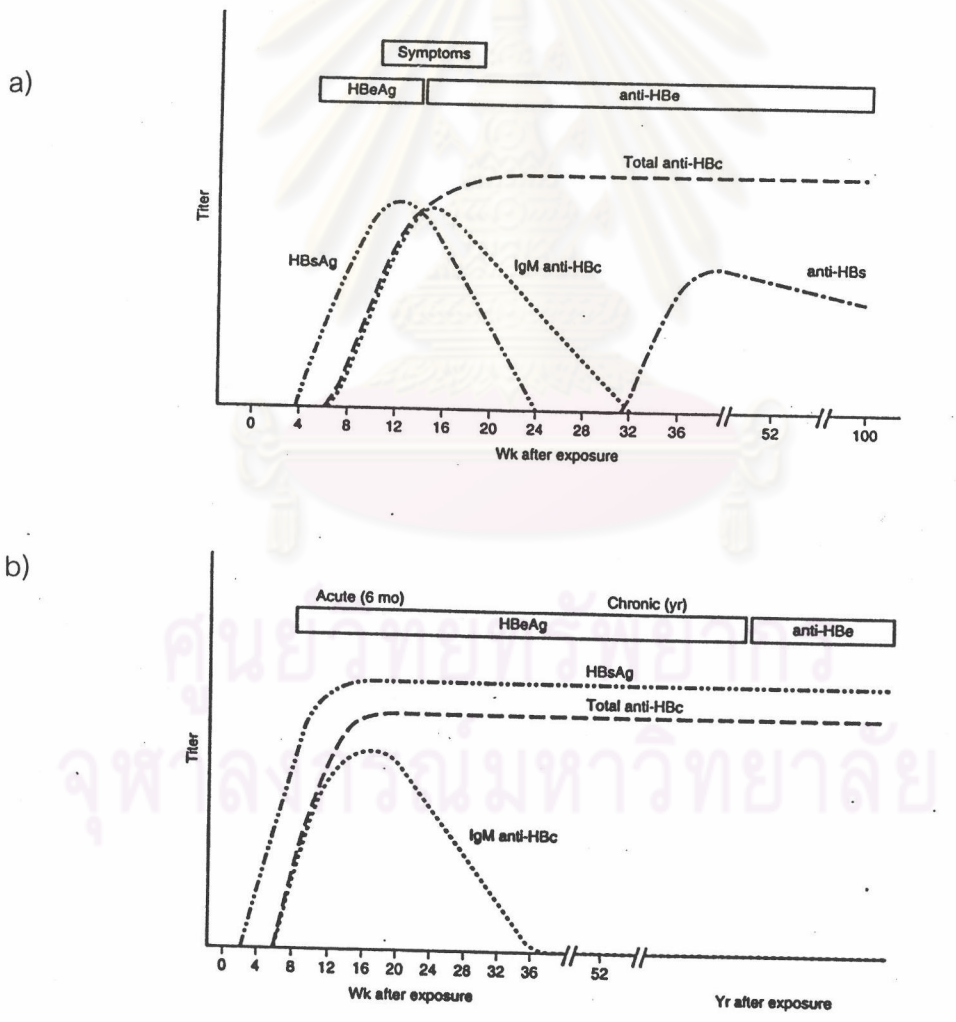

Figure 5 Characteristics of acute hepatitis B with recovery (a) and progression to chronic HBV infection (b) [16]

Transmission routes of HBV

There are different modes of HBV transmission: through a mixing of blood products, through sexual contact, perinatal from mother to fetus and horizontal transmission among young children. HBV is primarily found in the blood of infected individuals. Virus titres, as high as ten billion virions per millilitre of blood, have been reported in HBe-positive carriers. The virus is most efficiently transmitted through percutaneous introduction. Sexual transmission and perinatal transmission are less efficient, typically requiring high titres of virus. However, HBV has also been detected in other body fluids including urine, saliva/ nasopharyngeal fluids, semen, and menstrual fluids [40-41]. This virus has not been detected in feces, perhaps due to inactivation and degradation within the intestinal mucosa or by the bacterial flora [42]. Babies born to infected mothers are at very high risk of to becoming carriers and developing liver pathology [16].

The risk of HBV infection is increased based on certain behaviors. These include: injecting drug users, sexually active heterosexuals, men who have sex with men, infants/children in HBV endemic areas, infants born to infected mothers, health care workers, hemodialysis patients, people who have had more than one sexual partner in the last 6 months, those who received blood transfusions prior to 1975, hemophiliacs, patients and workers in institutions for the developmentally disabled, inmates at long term correctional facilities, and travelers to endemic areas. Uninfected individuals living with an HBV carrier are at greater risk of contracting HBV than those not living with a carrier. This is likely due to the fact that HBV can survive even on a dry surface for over a week [43].

The mechanisms of early childhood transmission in area of high endemicity are variable. Generally, infection clusters in household of persons with chronic infection [44]. The contribution of perinatal transmission to the overall burden of disease is related to the prevalence of HBeAg among pregnant woman. If a mother is HBsAg and HBeAg positive, 70 to 90% of her infants will become infected if not given immunoprophylaxis[45] Infants of HBsAg-positive but HBeAg-negative women are infected approximately 5 to 20% at birth. Anyway, infants who are not infected at birth are at increased risk of HBV

infection during early childhood because of household contact with HBsAg-positive mother [46].

Epidemiology of HBV

HBV is globally distributed among humans. The various strains of HBV are quite species specific. Though HBV has been found in other primates, humans remain the principal reservoir [47]. HBV accounts for a large portion of the disease burden worldwide with upwards of 350 million chronic carriers of the disease. The mortality rate of HBV infection approximately one million deaths per year indicates the extent of the global health problem posed by this virus [7].

The prevalence of HBV and its patterns of transmission vary throughout regions of the world, with about 45% of the world's population living in areas of high endimicity, defined as areas where 8% of the population has a positive test for the HBsAg. Additionally, another 43% of the world's population lives in areas of moderate endimicity (i.e. 2%-7% of the population is HBsAg positive). Only 12% of the world's population lives in areas of low endimicty (i.e., <2% of the population is HBsAg positive) (Figure 6) [48].

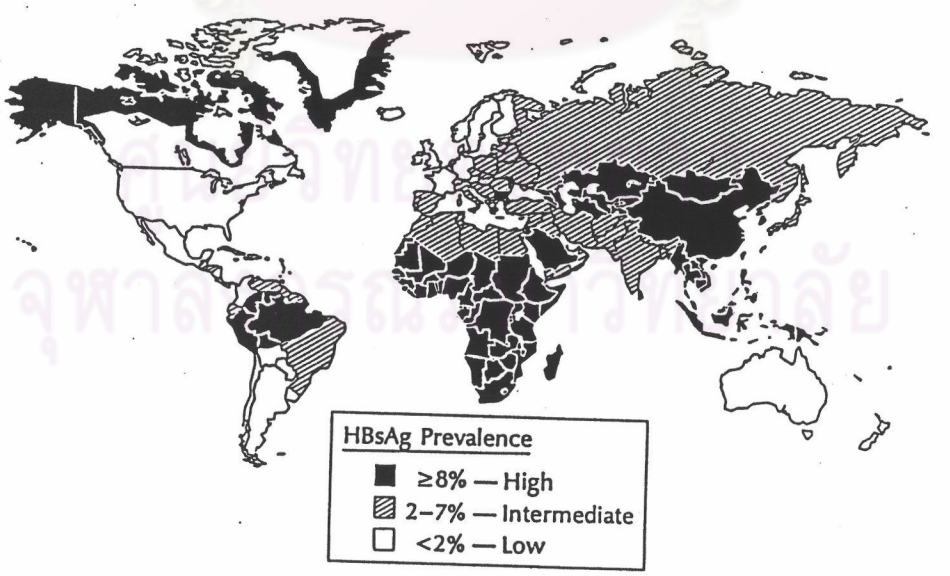

Figure 6 Geographic distribution of chronic HBV infection [48]

In areas of high endemicity, the life time risk of HBV infection is >60% and most infections occur at birth or during early childhood. when the risk of chronic infection is greatest. Because most early childhood HBV infections are asymptomatic, there is little recognition of acute disease, but rates of chronic liver disease and liver cancer are high. Areas of high endemicity include most of Asia (except Japan and India), most of the Middle East, the Amazon Basin of South America, most Pacific Islands groups, Africa and other special populations such as Native Alaskans, Australian Aborigines, and Maoris in NewZealand [49-51].

In East and Southeast Asian countries, as well as the Pacific, 35 to 50% of HBsAg-positive women are HBeAg positive. It is estimated that 3 to 5% of all infants in these countries may develop chronic HBV infection at birth and that up to 30 to 50% of all chronic infections among children may result from perinatal transmission [51].

Serotypes and genotypes of HBV

The serologic heterogeneity of hepatitis B virus has been established from immunodiffusion experiments. There are four serotypes which are based on subtypes of the hepatitis B surface antigen have been defined by two mutually exclusive determinant pairs d/y and w/r [52] .These subtypes are *adw, ayw, adr*, and *ayr* , where *a* is defined as the common determinant of all the subtypes.

With the description of four subdeterminants of *a*, later redefined as subdeterminants of *w* (*w1-w4*) at an international workshop in Paris in 1975 [53]. With the identification of the *q* determinant [54] the number of subtypes increased from eight to nine, due to the subdivision of the *adr* subtype into a q-positive and a q-negative category [55] . The nine serological subtypes are include *ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq+* and *adrq-* [56].

Since the different subtypes of HBV must be genetically defined by variations in the primary structure of the surface antigen, Magnius *et al.* (1995) reported the molecular basis identification for HBV serological variations using sequences of critical part of HBsAg as shown in Table 1 [56].

Table 1 Amino acid residues specification determinants of HBsAg

Position	Amino acid	Specificity
122	Lys (K)	*d*
	Arg (R)	*y*
127	Pro (P)	*w1*/w2*
	Thr (T)	*w3*
	Leu/Ile (L/I)	*w4*
160	Lys (K)	*w*
	Arg (R)	*r*

**w1* reactivity also required Arg122, Phe134 and/or Ala159 [56]

The HBV genotypes have been classified into six groups, designated A-F, based on an intergroup divergence of 8% or more in the complete nucleotide sequence [56]. These six different genotypes show a characteristic geographical distribution : genotype A is pandemic in North-West Europe, North America and Central Africa, genotype B is mostly found in Indonesia, China and Vietnam. Genotype C is most prevalence in East Asia, Korea, China, Japan, Polynesia and Vietnam; genotype D is predominant in the Mediterranean area and the Middle East extending into India. Genotype E is typical for Africa and genotype F is found in American natives and in Polynesia [56]. Recently, a new genotype of HBV, genotype G was reported by Stuyer *et al.* (2000)[57]. Sequence analysis, and subsequence phylogenetic analysis of the complete genome showed that this strain did not cluster with any of the known genotype.

HBV prevalence in Thailand

In the past, Asia and Southeast Asia has previously been classified as areas of high endemicity for HBV. It is estimated 50 million new cases of HBV infection are diagnosed annually. In Thailand, 8-10%of males and 6-8% females are HBsAg positive, with HBsAg also found in 30% of patients with cirrhosis and 50-75 % of those hepatocellular carcinoma [58]. The genotype and subtype prevalence in Thailand has been investigated by Theamboonlers *et al.* (1998) using PCR and sequencing of the *a* determinant, as well as the sub-determinants located on the *S* gene[59]. The results showed that genotype C (54.4%) dominates over genotype A (22.1%) and B1 (23.5%). The expected subtypes for Asia, adw2 (45.6%) and adr (54.4%), were reported[59]. Nowadays, the highly effective vaccination programs have shifted the high endemicity toward intermediate levels [60].

HBV infectivity : host and tissue tropism

Hepatitis B virus seems not to be readily absorbed or processed, replicated or spread in liver cells. When infecting a host organism and replicating within cells, viruses proceed through an orderly series steps. These steps include entry into the host organism, primary replication, spread to distant sites and secondary replication [61]. A successful viral infection can only be achieved if the infected cells provide an "appropriate environment" for viral survival and efficient development and propagation [62].

Virus attachment is also one of the important initial steps that determine among others host and organ tropism of viruses. Two basic mechanisms are considered and expected to be important in determining the tissue and host restricted replication of HBV. First, a molecular recognition by defined viral envelope domains of species and cell specific constituents expressed on the cell surface of the host (receptor theory). Second, involvement of hepatocyte-specific cytoplasmic and/or nuclear factors required for efficient propagation of HBV (theory of appropriate intracellular environment for viral survival and development) [62]. *In vivo* and *In vitro* experiments have shown that rat and

mouse hepatocytes are not susceptible to HBV infection [63]. However, cross-species replication of HBV has been obtained in hepatocytes of transgenic mice [64]. These findings suggest that the species specificity of HBV infection of hepatocytes is indeed determined and located at the initial steps of infection.

The receptor is often a key of viral tropism. Since amino acid sequences encoded by the *PreS* region are located exteriorly on the HBV envelope, it has been suggested that domains mediated host and tissue specific recognition are located within this sequence [9].

In 1979, Imai *et al.* reported a receptor for polymerized human and chimpanzee albumin on HBV particles co-ocurring with HBeAg[65]. Subsequent studies showed that the *PreS2* region contains the binding domain of artificially polymerized human serum albumins (pHSA) [66-67] and that liver cells bind to pHSA, although this binding is not species specific [68].

Furthermore, Neurath *et al.* (1986) demonstrated that an anti-serum against a PreS2 peptide (amino acid 1-24) was able to neutralize a defined HBV inoculum by showing that it was no longer infectious in experimentally challenged chimpanzees[69]. This finding suggested the PreS2 domain would act via pHSA as a organ specific attachment site of HBV. However, the importance of the pHSA binding was placed in doubt by the observation that pHSA was only able to bind if it was cross-linked by glutaraldehyde [70] or transglutaminase [71].

In addition, some observations suggested that the PreS1 domain, and not the PreS2 domain is probably the most important attachment site to liver cells. In 1986, Neurath *et al.*. found that suspended HepG2 cells bound to immobilized HBV particles and that peptide (amino acid residues 21-47) of PreS1 and antibodies against this peptide competed with or inhibited this binding[9]. The sequence around the binding site is highly homologous to a sequence of IgA and apparently binds to an IgA receptor of the liver [72]. The PreS1 attachment site recognizes a receptor not only on liver cells but also on monocytes and lymphocytes [73]. Furthermore, natural HBV inocula have been neutralized by rabbit antisera against PreS1 (residues 12-47) [74], and chimpanzees have been directly immunized by PreS1 at the same residues [75].

In view of fact that vaccination with major HBsAg protects well against HBV infection, major HBsAg was not considered as an important attachment protein of HBV. However, Leender *et al.* (1990) have recently found that major HBsAg was able to bind specifically to human hepatocytes, human fibroblasts and human blood mononuclear cells and supported the assumption that HBV attachment and penetration into human hepatocytes is mediated by specific receptors recognizing an amino acid sequence in the S region [(76)].

Recently, Annexin V (AnV), a Ca2+-dependent phospholipid binding protein presented on human liver plasma membranes, was identified as a specific SHBsAg binding protein [(77)]. The binding of SHBsAg to intact human hepatocytes can be inhibited by native or recombinant human liver Annexin V and by specific antibodies directed against human AnV. Despite 90% sequence homology, rat liver AnV does not bind to SHBsAg and does not inhibit the binding of SHBsAg to intact human hepatocytes, suggesting the existence of species specificity [(77)].

Following surface binding, virions are taken up into the hepatocyte, the mechanism of this uptake process for HBV is still unknown. Infection experiments in tissue culture systems have hampered the study of the replication cycle of this virus for long time. Recent development methods for the HBV infection were mentioned with three types of model systems :

1. Adsorption to human cells without the intention to detect penetration
2. Infection of primary hepatocytes
3. Infection of human hepatoma cell line

The limitations of the system of primary hepatocyte culture reside in the short supply of cells and the reproducibility of the infection assays. Gripon *et al.* (1988) introduced the use of dimethylsulfoxide (DMSO) and polyethylene glycol (PEG) to increase the susceptibility of HBV to human hepatocytes primary culture [(11)]. The effect of DMSO in improvement of virus production was confirmed by Galle *et al.* (1994) [(12)].

The study of HBV infection is hampered by the fact that the established human hepatocyte cell lines that retain the hepatocyte markers, such as HepG2 and Huh-7, are refractory to HBV infection. As the transfected HBV genome is transcribed efficiently in

these cell lines, attachment and entry are believed to be the restrictive steps. Paran *et al*.. (2001) demonstrated that DMSO-treated HepG2 hepatoblastoma cells are infected efficiently by serum derived HBV [78]. It is therefore possible that DMSO improves HBV infection by inducing expression and presentation of differential specific viral receptors.

Alternative way to increase the susceptibility of HBV into hepatocyte cells was increasing the specific viral binding receptors expression on the surface of host cells. Molecular biology techniques were used to express the recombinant protein on the cell surface of hepatocytes and lead them for HBV infection.

HBV Vaccine Development

A basic of vaccinology is that the prophylactic vaccine induces immunity. In terms of the HBV vaccine, the most important part of the genome is the *S* gene. It is particularly important because the S protein product contains an epitope that generates a protective immune response against the virus, and is thus key in the current HBV vaccine [16]. This epitope is dependent on the conformation of SHBs protein determined by cross-linked disulfide bonds. Thus, any effective vaccine for HBV must the production of anti-HBsAg IgG in the host.

Although the precise disulfide linkages between the cysteine residues of the envelope proteins remain undefined, the protein conformation endowed by the linkages determines the neutralizing epitopes. Antibodies to the common *a* determinants are found in the sera of immunized persons who remain protected against different subtypes of HBV, whereas antibodies to the subtype determinants do not confer protection [79]. The *a* determinant is located within the domains bordered by amino acid 120 and 147, which contain a predicted double loop structure projecting from the surface of the virus (Figure7). The major determinant for neutralizing antibodies is located predominantly on the second loops, between amino acids 139 and 147 [80-81].

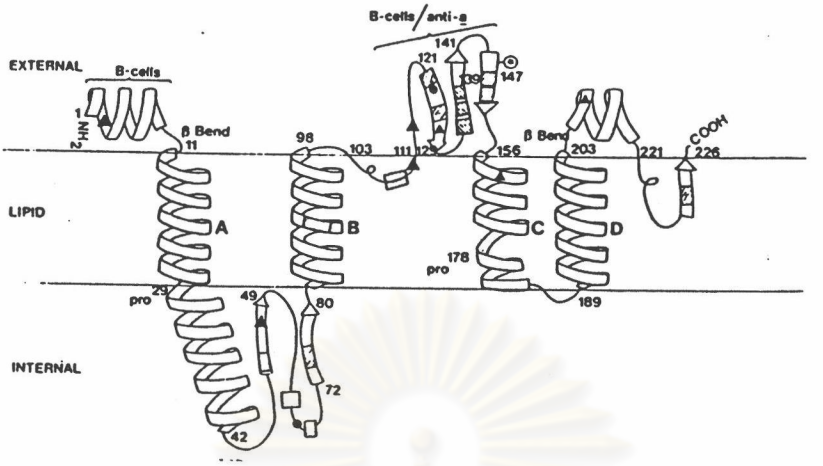

Figure 7 Schematic diagram of secondary structure of the HBsAg. Two separate external loop of the *a* determinant. The major epitope is located in the second loop between amino acids 139 and 147. The shaded areas indicate the locations of sequence variations for *w/r* (▲) and *d/y* (●) subtypes [81].

Vaccines against HBV were first made from the serum of people who had been HBV infected and subsequently cleared the infection. Because these individuals naturally produced anti-HBsAg IgG and then cleared the infection, they were immune to reinfection with HBV. The serum from these individuals was taken and purified antibody was passively administered to others in order to induce immunity. Although this strategy works in a theoretical manner, there are complications including supply shortage and host rejection of the foreign antibody[82].

In the early 1980's, Valenzuela *et al.* discovered a new way to produce this antibody utilizing recombinant DNA technology[83]. In an infected individual, HBsAg is present in two forms: as a protein on the surface of the 42 nm virion (also called the Dane particle) and as a secreted 22 nm particle that is a hollow sphere of surface antigen. Using the yeast *Saccharomyces cerevisiae*, researchers created a vector which contained the coding sequences for this surface antigen of HBV, HBsAg. The key to this success was that the yeast assembled this 22 nm protein in the same way that excess surface antigen assembles and is secreted in humans. Therefore, the artificial surface antigen resembled the naturally occuring particle [83].

One reason finding an alternate vector for expression of these surface antigen genes was so important in creating an effective vaccine was that this 22-nm particle could be administered safely to humans. Because the 22-nm particle contains the surface structure of HBV but does not contain the DNA of the actual virus, humans exposed to this product could produce their own anti-HBsAg IgG without any risk of being infected by HBV itself. Because the host was now producing its own antibody rather than passively receiving it from another individual, immunity was more likely to be long-lived and more effective. With the advent of recombinant DNA technology and the creation of the yeast vector, it was possible to create the first safe, effective recombinant vaccine for human use [84-85].

Shortly after the yeast vector was created, Michel Marie-Louise *et al.* (1984), researchers from France created a similar particle that could induce the correlate of immunity[86]. This time, the recombinant plasmid was transfected into Chinese hamster ovary (CHO) cells. Also, this plasmid was different from the recombinant yeast plasmid because it included an extra piece of the genome. While the yeast plasmid included only the *S* gene of HBV, the CHO clones used the *S* gene plus the *PreS2* portion of the genome, creating a vector that began one start codon upstream in the genome. In this case as well, the 22-nm particle containing the immunogenic HBsAg was produced by the artificial system. The researchers who created this vaccine compared its efficacy to the efficacy of HBsAg molecules purified from human systems, and found identical results. This event support to the idea that these artificial particles are as immunogenic as the naturally produced ones [86].

The two major producers of HBV vaccine containing the HBsAg that are available in the United States are Merck and SmithKline Beecham. In other parts of the world, there are several companies producing recombinant vaccines (such as Pasteur Merieux Connaught in France) as well as a few that are still selling plasma derived vaccinations. The two vaccines produced by Merck contain only the S portion of the genome, whereas the vaccine produced in France, GenHevac B, contains the PreS2 peptide as well. Yeast-based vaccines are most widely used. Today, the standard vaccine consists of three doses given intramuscularly in the deltoid muscles of children, adolescents, and adults, and in the anterolateral thigh muscle of children under two.

There has been a recent study in development of new hepatitis B vaccines made of the large and small hepatitis B surface proteins. The novel synthetic HBV envelope antigen vaccine, Hepagene B (Medeva), which contains *S*, *PreS1* and *PreS2* antigenic component, was produced in the mouse C127I clonal cell line [87-88]. The use of panel of monoclonal antibodies known to bind to epitopes within the common *a* determinant has demonstrated that the epitopes present on this new vaccine are comparable to those found with plasma-derived HBsAg. The anti-HBs levels elicited in chimpanzees protected them against an experimental challenge with HBV.

Other groups have looked into the case of chronic HBV carriers, those people who are unable to clear the viral infection more than 6 months after HBsAg appears in their serum. These researchers have found that chronic carriers are lacking a T-helper (Th) response to the virus. This lack of Th response is what may cause the lack of humoral response to this antigen. In order to investigate this problem, a cohort of chronic carriers was given the standard yeast recombinant vaccine to see if it had any therapeutic effect. Approximately 50% of those tested did respond to the vaccine and proceeded to clear the infection. Why the vaccine worked for some people and not the others is not clear. Theoretically, the vaccine exposes the host to the same immunogen that the natural virus does; it is the natural virus that the chronic carriers can not clear, yet somehow the vaccine had a therapeutic effect for some of these people but not others. The first question that was asked was whether or not therapeutic success of the vaccine had any correlation with specific MHC haplotypes. Although this would have provided a satisfactory explanation for the reactivity to the vaccine or lack thereof, this was not the case, and response or lack of response to the vaccine had no correlation to MHC haplotypes. At this point, it is not understood why 50% of chronic carriers responded to the vaccine while the other 50% did not [89].

Maternal and child HBV transmission and vaccination program

HBV is spread vertically, from mother to child. Combine this with the fact that age is inversely proportional to the risk of becoming a chronic carrier of HBV and neonates are a significantly affected population. This poses a threat that not only will the

baby develop liver problems, but as a chronic carrier also has a significant chance of passing the virus on to others horizontally. In fact, about 25% of neonates infected by their mothers at such a young age will develop liver failure or liver cancer. One-third of all chronic cases of HBV come from individuals affected as infants or young children. Because of these risks, it is especially important to take measures to prevent infection in neonates. In order to do so, it is recommended that all pregnant women be tested for HBV by the presence of HBsAg in their serum. If a mother is positive for serum HBsAg, her infant not only receives the standard vaccine, but within the first 12 hours after birth receives pre-formed anti-HBsAg antibody (immunoglobulin) in a form of passive immunization in addition to receiving the first dose of the standard recombinant vaccine. The second and third doses of the standard vaccine should be administered to these children on schedule, and in endemic parts of the world, a fourth dose is recommended at 12 months of age (6 months after the third dose). Thus, infants need to be vaccinated immediately in any case, and given immunoglobulin in addition if they are born to sero-positive mothers [90-92].

Nonhuman primate HBV

Apart from humans, hepadnaviruses have been isolated from several species, including the new world rodent woodchuck[93], the ground squirrel[94] and a range of bird species[95]. Up to now, nonhuman primate hepadnaviruses have been isolated from woolly monkey (*Lagothrix lagothrica*)[96], gibbon (*Hylobates spp.*)[3,97], chimpanzee (*Pan troglodytes*)[13], orangutan (*Pongo pygmaeus*)[15] and gorilla *(Gorilla gorilla)*[14]. These viruses all have similar genome structures and tissue tropisms.

Gibbon HBV was first isolated from a gibbon with acute hepatitis, which had been infected in the wild and was housed at the CDC for 2 years. The gibbon serum was positive for HBsAg but there was no recognition by an anti-preS1 mAb, which bound the preS1 hepatocyte receptor region, amino acid 27-35 [3]. Norder *et al.* reported the complete genome sequence of gibbon HBV in 1996 [97]. The complete genome of gibbon HBV (Genbank accession number U46935) was 3,182 nucleotides long and had a genetic organization identical to human and other mammalian

hepadnaviruses. The most divergent part of the genome, compared to other HBV strains, encodes for the Pre-S protein. Deletion of 33 base pairs at the start codon of *PreS1* was observed and a complete genome analysis showed that gibbon HBV was unique when compared to previously described HBV genotypes. It was most similar to the chimpanzee HBV strain that shared 90.3% nucleic acid homology at the level of the complete genome and 96.3% homology at the level of the *S* gene region corresponding to HBsAg. Thus, in most analyses, both the gibbon and the chimpanzee strains represented early lineages, indicating that these viruses were indigenous to their respective hosts and not recent acquisitions from humans [97]. Seroprevalence of captive gibbons housed at the International Center for Gibbon Studies (Santa Clarita, California) revealed that 47% (14/30) were positive for at least one marker of ongoing or previous infection with HBV [5].

The complete genome of gibbon HBV could be classified into four different phylogenetically distinct genomic group [14]. Not only reveal to chimpanzee viral strains, gibbon HBV is also clearly close relate to viruses isolated from orangutans and human genotpye C, which indicated the geographic clustering of viral spreading in Southeast Asia [15].

Woolly monkey hepatitis B virus (WMHBV) was isolated from a woolly monkey, the new world primate, with fulminant hepatitis. Analysis of nucleotide sequences of WMHBV showed that it is phylogenetically distinct from the human HBV and probably represents a progenitor of the human virus [96]. Supporting an intermediate host between human and rodents, only spider monkeys, the closest relative of woolly monkey showed the susceptibility to WMHBV infection while minimal replication was observed in a chimpanzee [96]. The survey of captive woolly monkeys revealed that approximately 50% of animals were positive for at least one marker of HBV infection [4].

The narrow host range, which has been an important restriction to experimental studies of HBV, is thought to be governed at the level of virus entry into cells. Little is know about the viral and host factors that account for the species-specificity of viral entry, however, the PreS1 domain at amino acid 12-20 , 21-47 and 82-90 were reported to be the ligand of viral binding [9,98].

Due to the close contact between human and captive animals, it was assumed that these animals were infected with human HBV[5]. Until recently reports, the nucleotide analysis of HBV recovered from wild-born chimpanzees shown the sharing genotype with captive chimpanzees [99]. This provided strong evidence of circulating HBV indigenous to chimpanzee in nature and might be support to the recent evidence for infection in the wild of others primates.

Fortunately, most of the nonhuman primates HBV contained the *a* determinant , the important region for protective immunity , with glycine at position 145 while other amino acid change were limited [100]. This indicated that available recombinant vaccine for human should prevent infection in the unlikely event of exposure to non-human primate virus and also used for prevent the neonatal transmission in nonhuman primates. Anyway, the protection efficiency of human vaccines to nonhuman viruses should be evaluated.

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