Chapter 2: Review of literature

2.1 HBV: INTRODUCTION

Hepatitis B virus (HBV) is a prototype member of the family Hepadnaviridae, and hepatotropic non-cytopathic DNA virus. Despite the presence of an effective prophylactic vaccine, it is estimated to infect 300 million people, with a particularly high prevalence in Asia and Africa [Lok & McMahon, 2001]. It is an enveloped virus and contains a circular partially double-stranded 3.2 kb in length DNA genome. HBV causes liver diseases that vary greatly in severity from person to person [Ganem & Prince, 2004]. Some subjects control infection efficiently and clear the virus from the bloodstream either without clinically evident liver disease or with an acute inflammation of the liver (acute hepatitis) that can resolve without long-term clinical sequelae. Other patients fail to clear the virus and develop chronic infection. Most chronically infected patients remain largely asymptomatic without life-threatening liver disease, but 10–30% develop liver cirrhosis with possible progression to liver cancer [Alberti et al., 1999; Lok & McMahon, 2001]. The rate of HBV chronicity is low in adults (5% or lower) but age and route of infection influence the outcome with exposure in neonatal life leading to a high rate of HBV persistence [Lok & McMahon, 2001; Ganem & Prince, 2004]. Outcome of infection and the pathogenesis of liver disease are determined by virus and host factors, which have been difficult to fully elucidate because the host range of HBV is limited to man and chimpanzees. The small genome encodes for four main genes by a series of overlapping reading frames. The polymerase gene is the largest open reading frame and it encodes for the multifunctional polymerase protein. The core gene encodes for core antigen (HBcAg) and the precore gene encodes for the hepatitis B e antigen (HBeAg), which has a large overlapping sequence with HBcAg. The three envelope

genes PreS1, PreS2 and S encode for the large, middle and small envelope proteins, respectively, and the X gene encodes for the accessory X protein.

HBV infection is the 10th leading cause of death and HBV related hepatocellular carcinoma (HCC) is the 5th most frequent cancer worldwide. About 30 percent of the world's population has serological evidence of current or past infection with HBV. Of these, an estimated 350 million are chronically infected with HBV and approximately 1 million persons die annually from HBV-related chronic liver diseases, including severe complications such as liver cirrhosis and HCC [Lok & McMahon, 2001].

The reservoir of HBV chronic carriers in the world is estimated at more than 200 million people and 80% of them reside in Asia and the western Pacific. In high-incidence areas, such as south-east Asia, perinatal transmission of HBV from carrier mothers to newborns appears to be the most important factor for the high prevalence of HBV infection and 70-90% of infants born to HBsAg/HBeAg-positive mothers become chronic carriers. Three possibilities of transmission of HBV from carrier mothers to newborns are suggested: (a) transplacental transmission in utero it was estimated that such transmission occurred in 5-15% of newborns; (b) transmission during delivery, which is considered the main mode of perinatal transmission; (c) postnatal transmission from mother to newborn, which is not common. HBeAg is the main maternal factor in determining whether infection of newborns will occur; the expression of this antigen seems to be determined genetically.

2.2 HBV INFECTION: EPIDEMIOLOGY

HBV is a hepatotropic non-cytopathic DNA virus that estimated to infect around 400 million individuals worldwide, with a particularly high prevalence in Asia and Africa [Lok & McMahon, 2005]. The prevalence of HBV carriers varies from 0.1 percent to 2 percent in low prevalence

areas (United States and Canada, Western Europe, Australia and New Zealand), to 3 to 5 percent in intermediate prevalence areas (Mediterranean countries, Japan, Central Asia, Middle East, and Latin and South America), to 10 to 20 percent in high prevalence areas (southeast Asia, China, sub-Saharan Africa) [Table no. 2.1 and Fig. 2.2] [J E Maynard et. al 2004]. The prevalence of chronic HBV infection in areas of high endemicity is at least 8%. As of 2010, China has 120 million infected people, followed by India and Indonesia with 40 million and 12 million respectively. According to WHO, an estimated 600,000 people die every year related to the infection [Alter, M. et.al 2008, revised fact sheet].

Routes of infection include vertical transmission (such as through childbirth), early life horizontal transmission (bites, lesions, and sanitary habits), and adult horizontal transmission (sexual contact, intravenous drug use). The primary method of transmission reflects the prevalence of chronic HBV infection in a given area. In low prevalence areas such as the continental United States and Western Europe, injection drug abuse and unprotected sex are the primary methods, although other factors may also be important [Custer; Sullivan, S et.al 2004]. In moderate prevalence areas, which include Eastern Europe, Russia, and Japan, where 2–7% of the population is chronically infected, the disease is predominantly spread among children. In high prevalence areas such as China and South East Asia, transmission during childbirth is most common, although in other areas of high endemicity such as Africa, transmission during childhood is a significant factor [Redd J. et.al, 2007]. In India, 1-4% of individuals are chronic carriers of Hepatitis B Virus (HBV). Infection with HBV may occur perinatally (vertical transmission), during early childhood (the so-called horizontal spread), through sexual contact or nosocomially. Our group has showed recently that the rate of intrauterine infection of HBV is 10%–79% and detection of any of the HBsAg, HBeAg, or HBV DNA in cord blood or

peripheral blood of neonates occurred in 78% cases with maternal HBV DNA levels more than 1.5 × 105 copies/mL as compared with 47% in cases with maternal HBV DNA levels 1.5×105 copies/ml (World J Gastroenterol 2004;10:437–4388; Pande et al. Paper presented at Digestive Diseases Week, 2008; abstract 252). According to the WHO report on prevention of HBV in India, HBsAg prevalence among general population ranges from 0.1% to 11.7%, being between 2% to 8% in most studies. HBsAg prevalence rate among blood donors ranged from 1% to 4.7%. With the exception of higher HBsAg positivity in some North Eastern states (~7%), no substantial geographical variation was apparent in other parts of India. Considering, on an average, HBsAg carrier rate of 5%, the total number of HBV carriers in the country is estimated to be about 50 million that forms nearly 15% of the entire pool of HBV carriers in the world and is the second largest pool of chronic HBV infections in the world [Martín-Ancel A. et.al 2004]. Using conservative prevalence estimates of different HBV seromarkers for estimating the number of HBV infections and serious disease outcomes in population, it was predicted that over 9 million are estimated to acquire HBV infection during their lifetime, an estimated 15, 07,000 will develop chronic HBV infection, and nearly 200,000 will die of acute or chronic consequences of HBV infection [Martín-Ancel A. et.al 2004], which clearly indicates an impending danger.

The wide range in HBV carrier rate in different parts of the world is largely related to differences in the age at infection, which is inversely related to the risk of chronicity. The rate of progression from acute to chronic HBV infection is approximately 90 percent for perinatally acquired infection, 20 to 50 percent for infections between the age of 1 and 5 years and less than 5 percent for adult acquired. The risk of infection in a child born to a Hepatitis B positive mother ranges from 10-85% depending on the mother's HBeAg status. It is observed that younger the age of

acquisition of HBV infection, higher the chances of becoming a chronic carrier. It is believed that as many as 90% of those who are infected at birth become chronic carriers and up to 25% of chronic carriers will die of chronic liver disease as adults. Infection with HBV is one of the most important causes of chronic hepatitis, cirrhosis of liver and hepatocellular carcinoma. These outcomes are all preventable by early childhood immunization. It is for this reason that the World Health Organization has recommended universal Hepatitis B vaccination.

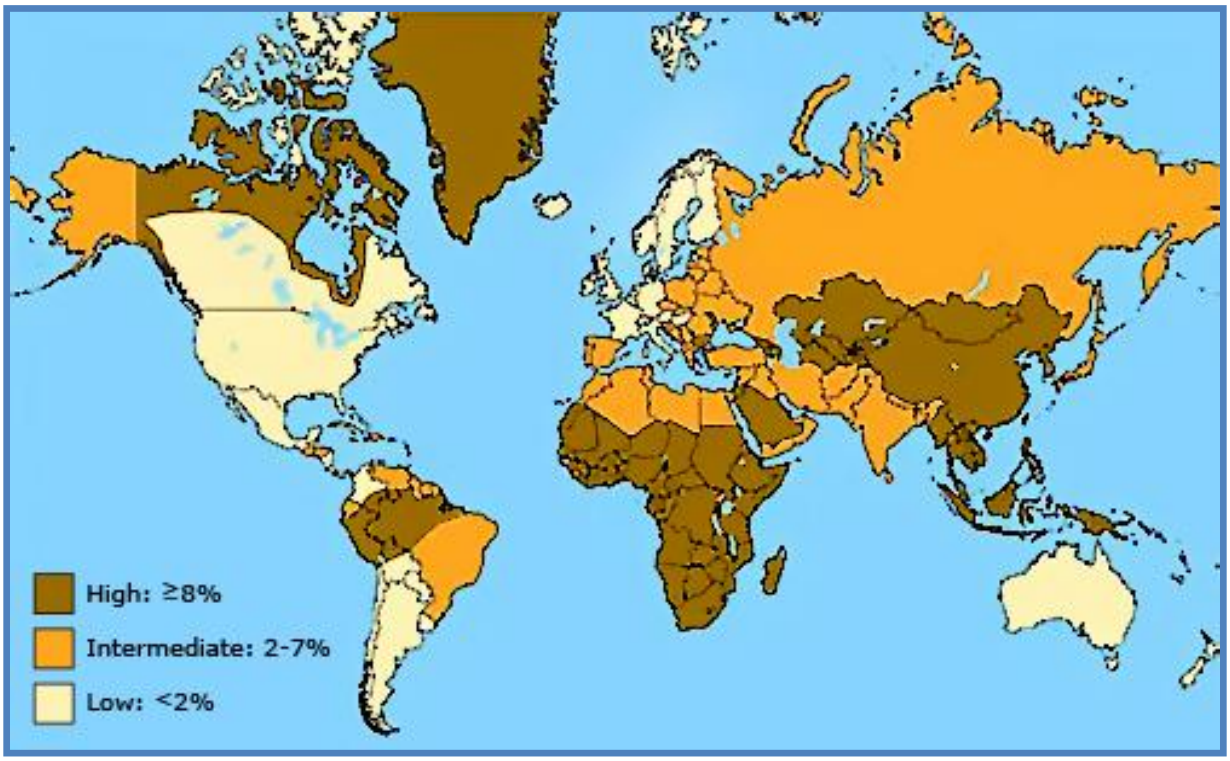

Fig.2.1 Geographic Distribution of hepatitis B virus infection [J E Maynard et. al 2004]

	High	Intermediate	Low
Carrier rate, percent	≥8 percent	2-7 percent	≤ 1percent
Geographic distribution	Southeast Asia; China; Pacific islands; sub-Saharan Africa; Alaska (Eskimos)	Mediterranean basin; eastern Europe; central Asia ; Japan; Latin and South America; Middle east	United States and Canada, western Europe; Australia; New Zealand
Predominant age at infection	Perinatal and early childhood	Early childhood	Adult
Predominant mode of infection	Maternal and infant Percutaneaous	Percutaneous; sexual	Sexual; Percutaneous

Table 2.1: Epidemiology and modes of transmission of hepatitis B virus infection

[Alter, M. et.al 2003]

2.3 HBV: STRUCTURE

HBV is an enveloped virus and contains a circular partially double-stranded DNA (full length negative strand and an incomplete plus strand) genome only 3.2 kb in length. The small genome encodes for four main genes by a series of overlapping reading frames. DNA genome consisting of approximately 3200 nucleotides, and the polymerase protein required for synthesis of viral DNA [Rehermann & Nascimbeni 2005].

The genome consist of four overlapping open reading frames; surface, core, polymerase and X genes. The polymerase gene is the largest open reading frame and it encodes for the multifunctional polymerase protein. By initiation of translation at three different in-frame initation codons in the pre S-S region the genome can encode three different proteins PreS1, PreS2 and S encode for the large (L), middle (M) and small (S) envelope proteins, respectively, and the X gene encodes for the accessory X protein (HBx). The core gene encodes for core antigen (HBcAg) and the precore gene encodes for the hepatitis B e antigen (HBeAg), which has a large overlapping sequence with HBcAg.

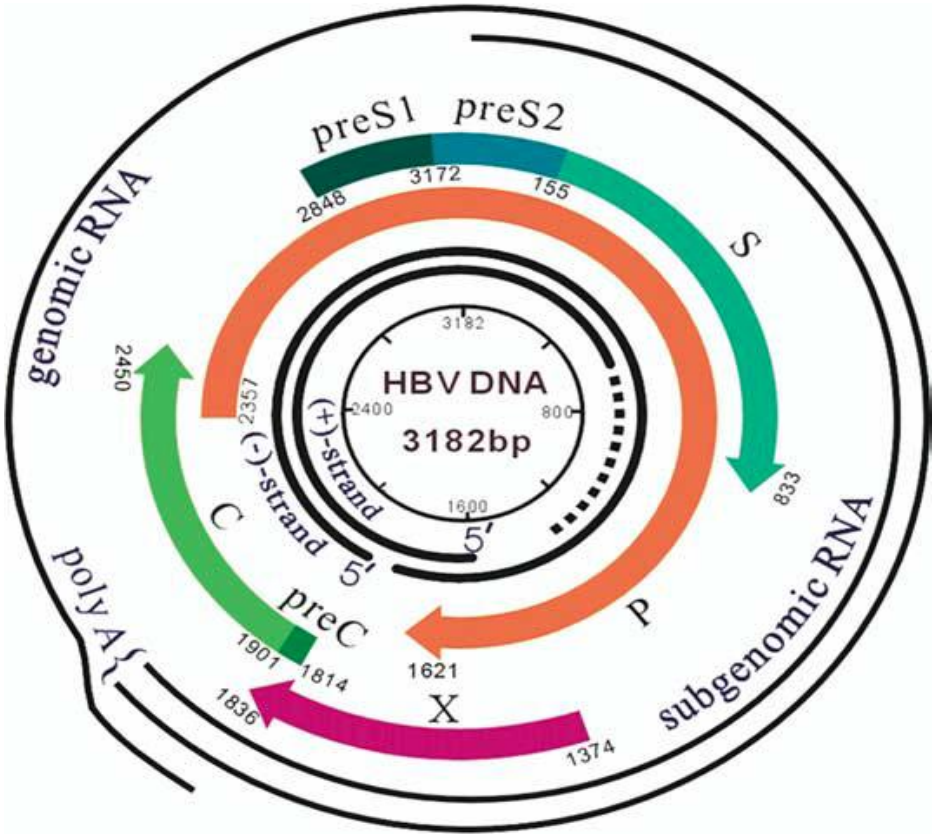

Fig 2.3 Structure of HBV genome: The genome of HBV is a double stranded circular DNA (3.2 kb), which contains four ORF coding for polymerase (P), surface antigens (PreS1, PreS2, and S), precore (preC), core (C), and X. [Xiaodong Zhang et.al J Lab Clin Med 2005]

2.4 HBV REPLICATION

After viral entry, the nucleocapsid is released into the cytoplasm and transported along the microtubules to the nuclear membrane. Nuclear import of the nucleocapsid through the nuclear pore complex is followed by disintegration of the nucleocapsid and liberation of HBV genome in the nuclear pore basket.

In the nucleus rc-DNA is converted into ccc-DNA which serves as the transcription template for all viral RNAs including pg-RNA as well as sub genomic RNAs. Two major transcripts of 3.5 and 2.1 and several minor transcripts are transcribed.3.5 transcript represents the message for viral core antigen and the polymerase serves as the template for reverse transcription for viral replication, therefore called the pg-RNA. 2.1 kb transcript represents the major transcript of s gene encoding the viral surface antigen. Two minor transcripts of 2.4 kb and .8 kb encode the large envelope protein and HBx protein. The pg-RNA is selectively packaged into progeny capsids and reverse transcribed into rc-DNA by viral polymerase capsids containing mature rc-DNA are then used for the assembly with the viral envelop in the ER leading to the formation of virions that will be released from the cell. Alternatively mature capsids can recycle rc-DNA to the nucleus and contribute to amplification of nuclear ccc-DNA pool [Christiene Neuveut et.al Journal of Hepatology, 2009]

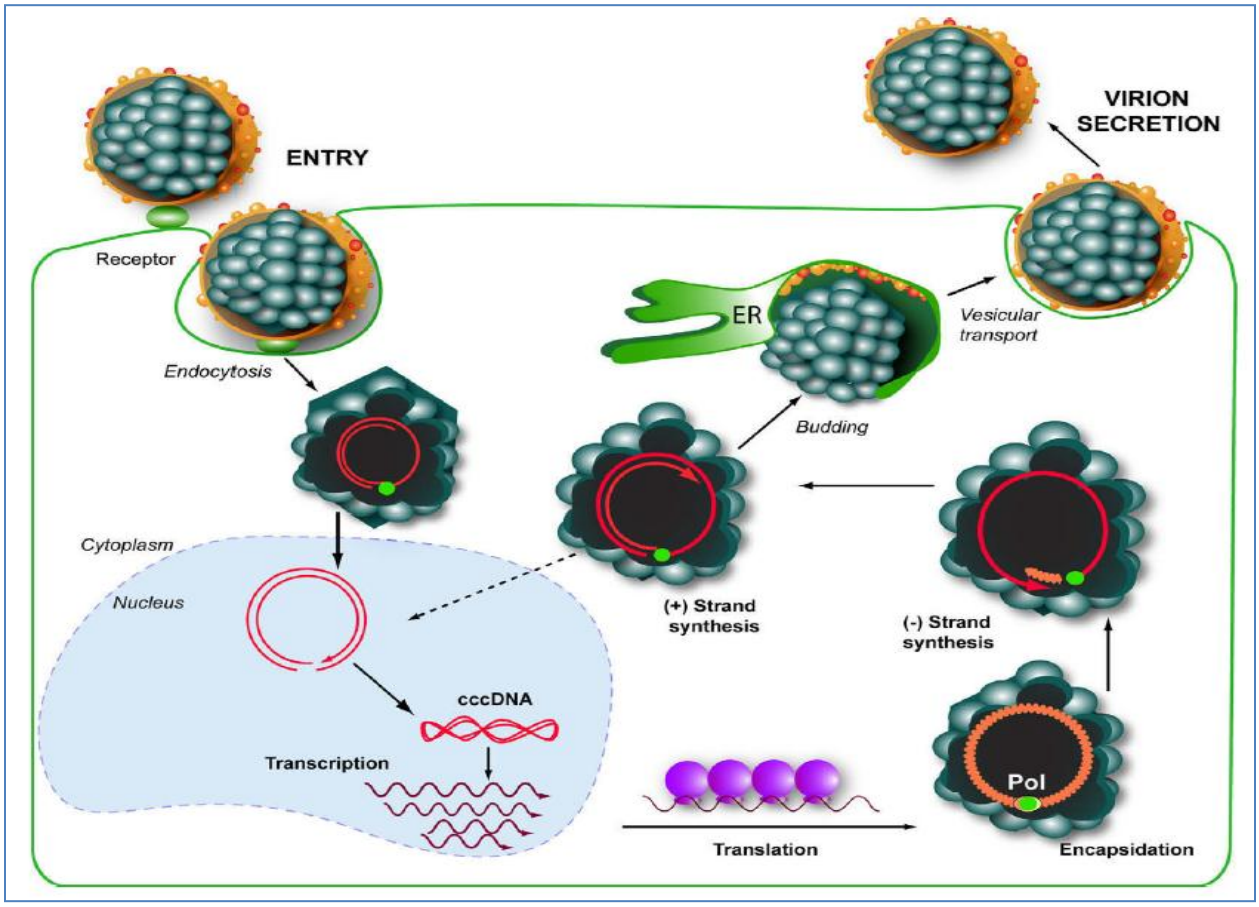

Fig. 2.4 HBV Replication: [Christiene Neuveut et.al Journal of Hepatology, 2009]

2.5 HBV INFECTION

Viral Hepatitis is a necroinflammatory liver disease of variable severity. Some subjects control infection efficiently and clear the virus from the bloodstream either without clinically evident liver disease or with an acute inflammation of the liver (acute that can resolve without long-term clinical sequelae. Other patients fail to clear the virus and develop chronic infection [Chisari FV et.al, 2000, Guidotti LG et.al, 2006].

Persistent infection by HBV is often associated with chronic liver disease that can lead to the development of cirrhosis and hepatocellular carcinoma (HCC). The type of cell-mediated responses expressed at the early stages of HBV infection can influence the subsequent outcome of hepatitis B. Indeed, recovery is associated with efficient activation of mechanisms of the innate immunity, which seem to be responsible for the early inhibition of viral replication [Asabe S. et.al, 2009]. A subsequent activation of HBV-specific T cells is probably crucial to complement the effect of the innate immunity and to allow complete control of virus replication. Moreover, control of infection cannot be achieved without rapid and efficient development of anti-envelope neutralizing antibody responses that is needed for elimination of free viral particles and inhibition of cell to cell spread of the virus [Wieland S.F.et.al, 2005]

In patients with chronic hepatitis B, HBV-specific T cell responses are weak or undetectable in the peripheral blood and T cells are attracted into the infected liver where they are diluted among virus non-specific T and non-T cells that are the predominant cell population of the intrahepatic infiltrate. The high viral and antigen load may be the main responsible for the T cell hypo responsiveness typical of chronic patients through exhaustion of T cell responses and expansion of T cells able to produce Th2 cytokines [Rehermann B. et.al 2000]. HBV is a typical non-cytopathic virus that can induce tissue damage of variable severity by stimulating a protective immune response that can simultaneously cause damage and protection, by curing intracellular virus through the destruction of virus infected cells [Huang C.F. et.al 2006]. Therefore, immune elimination of infected cells can lead to the termination of infection when it is efficient or to a persistent necroinflammatory disease when it is not. Destruction of infected cells, however, is not the only mechanism implicated in the elimination of intracellular virus, as demonstrated by studies carried out in animal models of HBV infection and in human hepatitis B showing the

importance of cytokine-mediated, noncytolytic mechanisms of anti-viral protection [Guidotti L.G. et.al 1999]. The first experimental evidence in favor of such mechanisms derives from studies performed in the transgenic mouse model. These studies showed that single-stranded and relaxed circular double stranded HBV-DNA replicative intermediates can be eliminated from the cytoplasm of HBV transgenic hepatocytes as a result of the anti-viral effect of IFN-α and TNF-α within the transgenic liver primarily by infiltrating HBV-specific CD8+T cells but also by CD4+ T cells [Mc Clarry H. et.al 2005].

Chronic HBV infection is largely mediated by HBV-specific T cells which play an important roles in inducing hepatocellular damage however some recent studies suggest that these cells often display functional impairment by T cell exhaustion via up-regulation of programmed death 1, Bim and other co-inhibitory receptors like CTLA-4. T cell impairment is even more pronounced in the livers of patients with chronic hepatitis B (CHB) versus their blood [Betoletti A. et.al, 2005, Chang J.J. et.al 2007]. Furthermore, activated HBV-specific CD8 T cells are often found to be present in the livers of patients without evident liver immunopathology, whereas non–virus-specific lymphocytes have usually massively infiltrated the livers of patients with hepatocellular damage. A model of HBV-transgenic mice has further confirmed that non–virus-specific lymphocytes can exacerbate the liver inflammation initiated by virus-specific CD8 T cells. These findings suggest that non–virus specific inflammatory cells infiltrating the liver may actively participate in HBV-associated liver pathogenesis [Thimme R. 2003].

Most chronically infected patients remain largely asymptomatic without life-threatening liver disease but 10–30% develops liver cirrhosis with possible progression to liver cancer [Alberti et al., 1999; Lok & McMahon, 2001]. The rate of HBV chronicity is low in adult infections (5% or lower) but age and route of infection influence the outcome with exposure in neonatal life

leading to a high rate of HBV persistence [Lok & McMahon, 2001; Ganem & Prince, 2004]. Outcome of infection and the pathogenesis of liver disease are determined by virus and host factors, which have been difficult to fully elucidate because the host range of HBV is limited to man and chimpanzees.

Mother to child transmission occurs often, either *inutero* or through exposure to blood or blood contaminated fluids at or around birth. Such perinatal transmission is believed to account for 35% to 50% of hepatitis B carriers. The risk of perinatal transmission is associated with the hepatitis B e antigen status of the mother. If a mother is positive for both hepatitis B surface antigen and e antigen, 70% to 90% of her children become chronically infected. If a mother is positive for the surface antigen but negative for the e antigen, the risk of transmission is significantly lower [Centers for Disease Control and Prevention - Accessed December 16, 2008].

Two types of vaccines for hepatitis B have been licensed. One is derived from plasma (plasma derived vaccine) and the other is derived from yeast or mammalian cells (recombinant vaccine). Repeated injections over months are required to mount an effective antibody response with vaccination. Hepatitis B immunoglobulin has high levels of antibody to hepatitis B surface antigen. The immunoglobulin is immediately effective and seems protective for several months, after which it wanes.

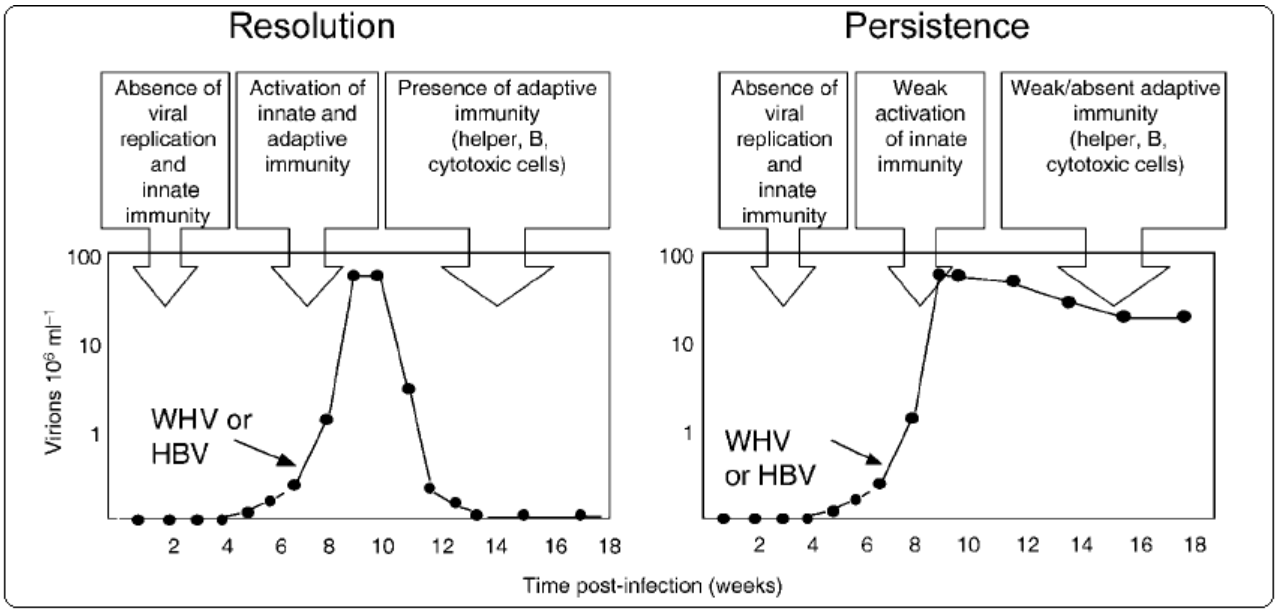

Fig. 2.5 Coordinate activation of innate and adaptive response is necessary for HBV control. [Guidotti et al. (1999); Thimme et al. (2003); Nakamura et al. (2001); Menne et al. (2002); and Cote et al. (2000)]

2.6 Clinical Course of HBV infection

Primary infection with HBV can be either symptomatic or asymptomatic. 95% of adults clear the infection and develop lifelong immunity, however in less than 5% of individuals, persistent infection is established which can progress to chronic hepatitis. 1% of individuals may develop fulminant hepatitis associated with large-scale tissue damage and may require a liver transplant with or without antiviral therapy [Dusheiko & Antonakopoulos 2008]. Approximately 20% of patients who develop chronic hepatitis progress to liver cirrhosis, and the chance of developing hepatocellular carcinoma are also increased by 100-fold [Beasley 1988]. The development of chronic hepatitis is much higher if infection is acquired by vertical transmission. Babies born to HBeAg positive mothers have a 70-90% risk of contracting the virus and 90% develop chronic hepatitis. If the mother is HBeAg negative however and is able to partially control viral

replication, the risk of infection is reduced to 10-40% and rate of chronicity is also reduced (40-70%) [Alter 2003].

Chronic infection has a dynamic course which varies greatly between patients. Patients may be divided broadly into 3 categories of disease; HBeAg positive CHB, HBeAg negative CHB or HBeAg negative inactive carrier state [Dusheiko & Antonakopoulos 2008]. HBeAg positive patients are characterized by a high level of HBV replication sustained over a long period of time. They may or may not have raised ALT. Individuals who are infected at birth go through an immunotolerant phase, characterized by elevated HBV DNA (>105 copies/ml) and presence of HBeAg in the serum, in the absence of liver inflammation. This may be due to HBeAg mediated tolerance of the immune response, which subsequently fails to control HBV replication, and does not mediate damage to HBV infected cells via cytolytic killing which would normally cause liver inflammation. In most patients, there is a decline of serum viral load titer over time, and HBeAg to anti-HBe seroconversion occurs at a rate of 5-10% per year. As mentioned above, HBeAg negative patients may fall into two groups. The first group is patient with anti-HBe positive chronic hepatitis. In these individuals, absence of HBeAg from the serum is due to viral mutations in the pre C region which prevents its expression. These patients are susceptible to active flares of liver disease associated with acute increases of HBV DNA and ALT. The second group have seroconverted to anti-HBe status, and typically have normal ALT and HBV DNA<10^5copies/ml [McMahon2005].

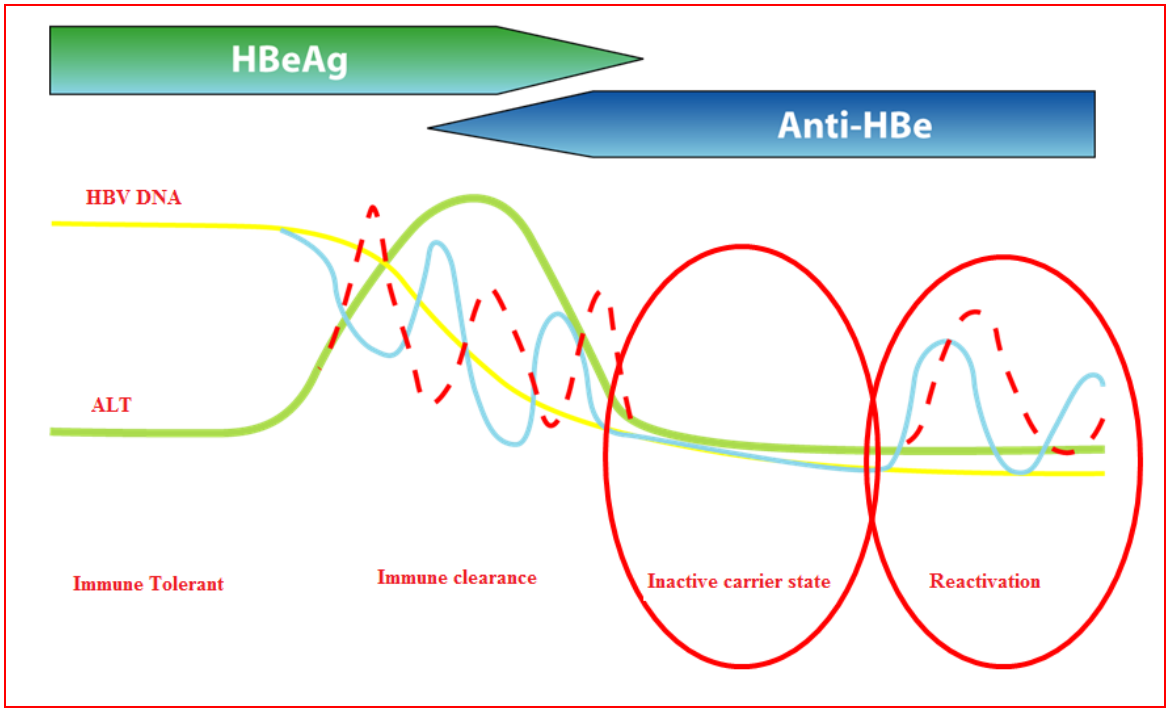

Fig.2.6(A) Phases of HBV infection,Yim J.Y. et.al 2006

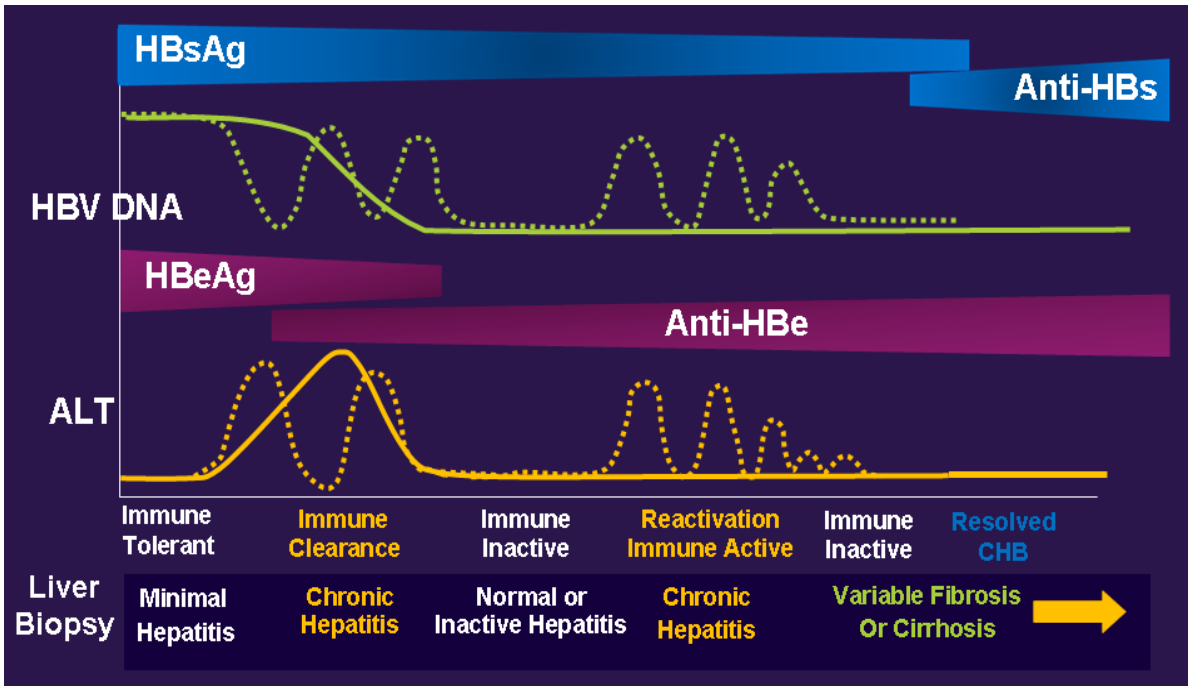

Fig. 2.6 (B) Natural history of chronic HBV infection [adapted from Zakim and Boyer, Hepatology (A text book of liver disease, 5th edition)]

2.7 HEPATITIS B VIRUS VACCINATION

Despite advances in antiviral therapy, only a minority of patients with chronic hepatitis B will have a sustained response. Thus, primary prevention by vaccination to increase herd immunity remains the main thrust in the control of hepatitis B virus (HBV) infection. A safe and effective vaccine against the hepatitis B virus surface antigen has been available since 1982 [Lavanchy 2004]. Hepatitis B vaccine is effective not only in preventing HBV infection but also in preventing the sequelae of chronic HBV infection. Its efficacy has been shown in countries like Taiwan where introduction of the vaccine into routine immunization schemes has reduced the prevalence of HBV infection by 93% in under 15 year olds [Namgyal 2003].

Three different classes of hepatitis B vaccine are available based upon how they are derived (from plasma, yeast, or mammalian cells). Currently available hepatitis B vaccines are extremely safe and have an efficacy of >90 percent and are effective against all HBV serotypes and genotypes. The development of Hepatitis B vaccine is considered to be one of the major achievements of modern medicine. Thus, HBV infection can potentially be eradicated through global vaccination.

However, despite its success and a recommendation by the WHO in 1991 that all countries implement the vaccine into their routine immunization schemes by 1997, overall uptake has been poor (116/215 countries took part in 2000), due to a number of limitations. Unfortunately, due to the lack of funding and infrastructure to purchase and deliver the vaccines, vaccination coverage is low in many developing countries and also low in many developed countries due to the misconception that vaccination is only necessary in high-risk groups in non-endemic areas. Based upon the WHO/UNICEF immunization coverage estimates as of August 2007, the Hepatitis B vaccine coverage globally was estimated at 60 percent, with almost 90 percent in the

Americas, decreasing to 49 percent in Africa and 28 percent in Southeast Asia, the region that requires it most [World Health Organization, Immunization, www.who.int/vaccines/en/hepatitisb.shtml, 2006].

Another major obstacle is that 5 to 10 percent of individuals do not respond to currently available vaccines. Thus, public health education and infection control measures to interrupt transmission of HBV is important. There is no doubt that the development of hepatitis B vaccines is a major accomplishment in modern medicine. Nevertheless, much work remains to achieve the goal of global eradication of HBV infection. Clearly, the costs of vaccines have to be reduced and the infrastructure for their delivery has to be simplified.

Although new emerging drugs and treatment regimes may successfully suppress HBV viral load for longer and with fewer side effects or further clinical sequelae, it is unlikely that this therapy alone will provide a definitive cure which can be delivered cheaply and efficiently to millions of individuals in developing countries suffering with chronic infection today. Clearance of viral infection and disease resolution will probably require a synergistic boost to the host's own immune response, and this approach in addition to suppression of virus load with antiviral agents, may tip the balance in favor of viral clearance. There is a great need therefore to better understand the mechanisms of disease and why the immune system fails in some, but not other individuals. This knowledge will facilitate development of novel immunotherapeutic strategies to aid viral clearance and reduce the global burden of disease- the ultimate aim of research into viral infection.

Neonates of HBV positive mothers — Hepatitis B vaccine is recommended for all neonates of HBsAg positive mothers, and in many countries is also recommended for neonates of HBsAg negative mothers. [Margolis HS et.al, JAMA, 2005]. Vaccination of neonates of HBsAg positive

mothers is the most important step toward the eradication of chronic HBV infection. The standard regimen consists of passive and active immunization. Hepatitis B Vaccine and Hepatitis B immunoglobulin (HBIG) are given at the same time at two different sites within 12 hours of delivery. The neonates then proceed to two additional doses at months 1 to 2 and months 6 to 12.

2.8 Immune response to HBV Infection

2.8.1 Innate Immune Response

Innate immunity generally plays a role immediately after infection to limit the spread of the pathogen and initiate efficient development of an adaptive immune response. The innate response is initiated almost immediately after inoculation, and aims to rapidly contain viral spread. The hallmark of this response is production of Type I IFNs, which can inhibit viral infection but also have immunoregulatory effects including induction of NK cell function [Lanier 2008], Dendritic cell maturation [Dalod et al. 2003] and priming of CD8 T cell responses [Tough et al. 1996]. Production of other cytokines such as IL-12, IL-6 and TNF may also contribute to viral control [Pichlmair & Reise Sousa 2007]. NK cell IFN-γ production or direct lysis of infected cells too is important during the innate response. These cells may recognize altered levels of MHC-I on infected cells, or become activated by stress-induced molecules [Moretta et al. 2005].

The general pattern of fast viral spread and subsequent rapid activation of innate immunity has been deduced primarily from mouse models of different viral infections. Lymphocytic choriomeningitis virus (LCMV) and murine cytomegalovirus [Biron, 2001; Ou et al., 2001] and holds true for many human viruses like Human immunodeficiency virus, Cytomegalovirus and

Epstein–Barr virus. However, the simple observation of clinical, virological and immunological phenomena that follow HBV infection depicts a completely different and unconventional pattern.

Experimental data collected, mainly in animal models but also in humans [Fong et al., 1994], show that after inoculation, HBV does not immediately start to replicate efficiently. HBV-DNA and HBV antigens are not detectable in serum or the liver until 4–7 weeks post-infection [Berquist et al., 1975; Korba et al., 1989; Fong et al., 1994; Guidotti et al., 1999; Thimme et al., 2003]. Following this period, HBV begins a logarithmic expansion phase that can be detected in the liver and serum, reaches levels of 109–1010 copies/ml [Whalley et al., 2001] and infects most hepatocytes [Jilbert et al., 1992; Kajino et al., 1994; Guidotti et al., 1999; Thimme et al., 2003]. Rigorous experiments in chimpanzees showed that while HCV replication in the liver starts immediately after infection [Thimme et al., 2002], larger doses of HBV inoculums do not enter an exponential phase of replication until 4–5 weeks after infection [Thimme et al., 2003]. The initial lag phase of HBV replication does not appear to be a consequence of HBV inhibition by elements of innate and adaptive immunity. The activation of IFN-γ, interleukin (IL-2) and tumor necrosis factor (TNF)-a and intrahepatic recruitment of inflammatory cells is delayed until the logarithmic expansion of HBV in experimentally infected woodchucks [Cote et al., 2000; Hodgson & Michalak, 2001; Nakamura et al., 2001] and chimpanzees [Guidotti et al., 1999]. Furthermore, a recent elegant paper by Wieland et al. longitudinally analyzed the activation of cellular genes in three experimentally infected chimpanzees. In all three animals, no cellular genes were activated within the liver during the lag phase of infection, confirming that intrahepatic activation of innate immunity did not affect initial HBV spread [Wieland et al., 2004]. The causes of the delayed appearance of quantifiable levels of HBV proteins and HBV-DNA in the first weeks of infection are not clear.

HBV might initially infect very few hepatocytes and spread with a relatively slow doubling time. Alternatively, we can speculate that immediately after infection, HBV does not reach the liver, but remains in other organs. Interestingly, longitudinal virological analysis of woodchuck hepatitis virus (WHV) infection showed that the initial site of WHV infection was not the liver but the bone marrow [Coffin & Michalak, 1999]. However, the lymphotropism of WHV seems more pronounced, diffuse and with pathological importance than HBV [Coffin & Michalak, 1999; Lew & Michalak, 2001], and thus this possibility is attractive but still speculative in HBV infection.

A further characteristic of HBV in relation to early host defence mechanisms resides in the lack of IFN-a and b production. HBV replication can be efficiently limited by a and b IFN [McClary et al., 2000; Wieland et al., 2000], but data on acutely infected chimpanzees suggest that such antiviral cytokines are not triggered by HBV replication [Wieland et al., 2004]. HBV might have evolved strategies to escape the initial antiviral defence mechanisms activated by the Toll-like receptor system. It has been proposed that because HBV replicates within nucleocapsid particles, viral replicative intermediates of single-stranded RNA or viral DNA, generally strong activators of type I IFN genes [Lund et al., 2003; Heil et al., 2004], are protected from cellular recognition [Wieland et al., 2004].

Hepatitis, after HBV infection, is generally mild in chimpanzees compared with humans and it is possible that the inability to detect activation of genes related to innate immunity is a reflection of the mild profile of disease. Still, the striking difference between the early detection of type I IFN activation during early phases of HCV infection in chimpanzees [Bigger et al., 2001; Su et al., 2002] and its absence in HBV-infected animals is a further indication of the ability of HBV to sneak through the front line host defence mechanisms. Such early events are difficult to

analyze during natural infection in humans. HBV-infected patients are mainly detected after onset of clinical symptoms, which occur well after infection (10–12 weeks) [Webster et al., 2000]. Nevertheless, it is interesting to note that the lack of early symptoms in HBV infected patients such as fever and malaise, which is characteristic of other human viral infections, constitutes indirect evidence of the defective type I IFN production during the early phases of HBV infection.

2.8.2 The adaptive immune response

The antibody response

The antibody response to the HBV envelope antigens is a T cell dependent process [Tsui LV et.al 1995, Chang J.J. et.al. 2008]. Because these anti-envelope antibodies are readily detectable in patients who clear the virus and recover from acute hepatitis, and they are usually undetectable in patients with chronic HBV infection, they are thought to play a critical role in viral clearance by complexing with free viral particles and removing them from circulation or by preventing their attachment and uptake by hepatocytes. This notion is supported by the observation that chimpanzees that resolved a previous infection are completely protected from rechallenge [Moss B et.al 1984, Bertoletti A. et.al 2006]. The appearance of neutralizing antibodies, however, occurs relatively late after HBV exposure and, thus, it is unlikely to contribute to the early phase of viral clearance during acute infection. Instead they probably prevent viral spread from rare cells that remain infected after resolution of HBV infection.

The CD4 T cell response

The peripheral blood CD4 T cell response to HBV is vigorous, and multispecific in patients with acute hepatitis who ultimately clear the virus, while it is relatively weak in persistently infected patients with chronic hepatitis [Ferrari C et.al 1990]. Although the association between a strong

CD4 T cell response, acute hepatitis and viral clearance suggests that a relationship exists between these events [Tsui LV et.al 1995, Ferrari C et.al 1990, Jung MC et.al 1991], CD4 T cell depletion at the peak of HBV infection had no effect on viral clearance and liver disease in infected chimpanzees, suggesting that CD4 T cells do not directly participate in viral clearance and tissue damage. As we will discuss in more detail later in this review, CD4 T cells probably contribute indirectly to the control of HBV infection by facilitating the induction and maintenance of the virus-specific B cell and CD8 T cell response.

The CD8 T cell response

The HBV specific CD8 T cell response plays a fundamental role in viral clearance and the pathogenesis of liver disease. A vigorous polyclonal CD8 T cell response is readily detectable in the peripheral blood of patients with acute hepatitis, who ultimately clear HBV. In contrast, the peripheral blood CD8 T cell response in chronically infected patients is weak and narrowly focused

[Bertoletti A et.al, 2008]. The livers of these patients contain virus-specific CD8 T cells that likely contribute to disease pathogenesis, but for functional and/or quantitative reasons are unable to clear the infection. Interestingly, a recent study that examined a relationship between the number of intrahepatic HBV specific CD8 T cells, extent of liver disease, and levels of HBV replication in chronically infected patients indicated that inhibition of virus replication could be independent of liver damage, and that the functionality of HBV-specific CD8 T cells was more important than the number of T cells to control HBV replication [Maini MK. et.al, 2000]. Experiments in chimpanzees have shown that the viral clearance and the onset of liver disease coincide with the accumulation of virus-specific CD8 T cells and the induction of interferon gamma (IFNg) and IFNg-inducible genes in the liver [Thimme R et.al, J Virol 2003].

Importantly, depletion of CD8 T cells at the peak of viremia delays viral clearance and onset of viral hepatitis until the T cells return, proving that the viral clearance and liver disease are mediated by virus specific CD8 T cells.

During initial infection, naive T cells are primed by antigen, co stimulation and inflammation and differentiate into effector T cells. Clearance of infection and antigen allows a subset of these functional effector T cells to further differentiate into highly polyfunctional memory T cells able to coproduce many cytokines (such as IFN-γ, tumor necrosis factor (TNF) and IL-2), becoming cytolytic and proliferating vigorously (top). These cells also have considerable survival capacity and are maintained long term without antigen. During chronic infection (bottom), infection persists after the effector phase. As antigen and/or viral load increases, T cells progress through stages of dysfunction, losing effector functions and other properties in a hierarchical manner. T cell exhaustion is also accompanied by a progressive increase in the amount and diversity of inhibitory receptors expressed. In addition, altered inflammation and changes in immunoregulatory cytokines such as IL-10 and/or TGF-β can have an increasingly important role. Ultimately, if the severity and/or duration of the infection is high and/or prolonged, virus-specific T cells can be completely eliminated, leading to loss of virus-specific T cell responses

2.9 Mechanisms of HBV clearance

It is widely believed that the CTL response clears viral infections by killing infected cells. CTL killing is an inefficient process, however, requiring direct physical contact between the CTLs and the infected cells. Thus, it may not be possible for CTLs to kill all HBV infected cells if the CTLs are greatly outnumbered as occurs during HBV infections in which as many as 10^{11} hepatocytes can be infected [Thimme R et.al, J Virol 2003]. Thus, although the liver disease in HBV infection is clearly due to the cytopathic activity of the CTL response, viral clearance may require more efficient CTL functions than killing. Important insights into the pathogenetic and

noncytopathic antiviral functions of the CTL response have come from studies in HBV transgenic mice that develop an acute necroinflammatory liver disease after adoptive transfer of HBsAg specific CTL clones. In that model, the CTLs rapidly enter the liver and recognize viral antigen that triggers two events: (a) apoptosis of the hepatocytes that are physically engaged by the CTLs, and (b) secretion of IFN g, which noncytopathically inhibits HBV gene expression and replication in the rest of the hepatocytes [Guidotti LG et.al, Immunity 1996,] by preventing the assembly of HBV RNA-containing capsids in the cytoplasm in a proteasome-and kinase-dependent process. During this remarkable process, the viral nucleocapsids disappear from the cytoplasm of the hepatocytes and the viral RNAs are destabilized by a SSB/La-dependent mechanism in the nucleus, yet the hepatocytes remain perfectly healthy [Guidotti LG et al. PNAS, 1994, Tsui LV et.al PNAS, 1995]. Antibody blocking and knockout experiments in the HBV transgenic mouse model further demonstrated that the cytopathic and antiviral functions of CTLs are completely independent of each other. These results suggest that a strong intrahepatic CTL response to HBV can suppress viral gene expression and replication noncytopathically.

2.10 Mechanisms of chronic viral persistence

For a noncytopathic virus like HBV to persist it must either overwhelm or not induce an effective antiviral immune response or it must be able to evade it [Milich DR et.al Proc Natl Acad Sci U S 1990]. Data from transgenic mice indicate that neonatal tolerance to HBeAg is a crucial mechanism responsible for the lack of an antiviral immune response following mother to infant transmission [Chen MT et.al, 2004], In this context, a cytokine imbalance oriented towards preferential Th2 responses, as a result of the capacity of Th2 cells to evade tolerance more

efficiently than Th1 cells, has been suggested to favor chronic evolution of HBV infected neonates [Brunetto MR et.al, Proc Natl Acad Sci U S A 1991]. The immunological basis for viral persistence during adult onset infection is not well understood. It is well known that the T cell response is much less vigorous in chronically infected patients than it is during acute infection [Bigger CB et.al J Virol 2001]. However, this T cell hypo responsiveness is more likely to be the consequence of the active viral replication in persisting chronic infections rather than the primary cause of viral persistence. Peripheral tolerance or exhaustion of the T cell response by the high viral load that characterizes most persistently infected patients can be its cause. In support of this possibility, recovery of HBV-specific CD4 and CD8-mediated responses has been observed in viremic HBeAg+ patients following the decline of viremia caused by lamivudine treatment [Webster GJ et al. J Virol 2004, Reignat S, et al. J Exp Med 2002].

Several observations indicate also a role for soluble HBeAg in the down-regulation of HLA class II restricted T cell responses. Immunization of mice with HBeAg can favor the production of Th2 cytokines [Zoulim F, et.al, J Virol 1994] which in turn can cause suppression of Th1 production. In the transgenic mouse model, circulating HBeAg can also delete Th1 cells by Fas-FasL-mediated mechanisms, leading to predominance of Th2 cells [Zhang Z et.al, J Clin Invest 2001]. Given the role of Th1 cytokines in the non cytolytic control of virus replication, a T cell response skewed towards a prevalent Th2 profile may contribute to viral persistence. These findings raise the question of whether and how a Th1/Th2 imbalance actually occurs at the early stages of HBV infection when the most relevant pathogenetic events determining the outcome of HBV infection are believed to take place. Although definitive demonstrations are still lacking, several candidate mechanisms have been proposed to contribute to HBV persistence. First, there is some evidence that privileged sites may play a role since HBV can infect extrahepatic tissues.

Moreover, in HBV transgenic mice that express the virus in the liver and the kidney, circulating HBV specific CTL can cause hepatitis, but not nephritis, due to the limited access of the CTL to antigen positive cells present on the other side of microvascular barriers that exist in the kidney but are not present in the liver sinusoid. Second, hepatocytes can be induced to express Fas ligand during an inflammatory response [Sallusto F et.al, 1999] and HBV itself might be able to induce Fas ligand expression by the hepatocyte. Since infected cells that express Fas ligand can protect themselves against CTL -mediated injury by actively destroying the CTL via the same Fas ligand-Fas receptor pathway that CTL use to kill their target cells [Steinman RM et.al., Hum Immunol 1999], individuals with the highest hepatocyte expression of Fas ligand could delete their HBV-specific CTL more efficiently and, therefore, could have an higher probability to become chronically infected. All of these theories are testable, but they are strictly speculative at present.

Third, virus-specific CTL that might otherwise become activated by antigen recognition in the immunostimulatory context of secondary lymphoid organs might be inactivated if antigen is presented in the absence of co stimulatory signals in the liver. In this context, presentation of soluble antigens by liver endothelial cells has been shown to induce specific T cell tolerance [Bertolino P et.al, Immunol Cell Biol 2002]. Fourth, HBV envelope antigens represent an exception to the rule that CTL activation is selectively induced by recognition of endogenously synthesized antigen, because also exogenous forms of HBV envelope proteins can gain access to the class I pathway of antigen processing and presentation [Crispe IN, et.al, Nat Rev Immunol 2003, Isogawa M et.al, Immunity 2005]. CTL activation by this mechanism has been suggested to cause selective killing of HBV envelope-specific B cells acting as antigen presenting cells. Suppression of the anti-envelope antibody response might ensue, facilitating chronic evolution of

HBV infection [Crispe IN, et.al, Nat Rev Immunol 2003]. Additionally, viral mutations that abrogate or antagonize antigen recognition by virus specific T cells have been reported in the context of strong and narrowly focused CTL responses in patients with chronic HBV infection [Kremsdorf D et.al, Oncogene 2006]. In view of the multispecificity of the CTL response in the acute phase of HBV infection, current data favor the notion that selection of CTL escape mutants is most likely to occur after a persistent infection is already established. In this setting, viral mutations could solidify the chronicity of the infection and perhaps even make it irreversible. Whether such mutations can also serve as the primary cause of viral persistence in the context of a multispecific T cell response remains to be proven.

2.11 Regulatory T cells (CD4+CD25hi FOXP3+)

Studies of numerous experimental models have provided evidence that a population of specialized T cells is able to regulate the immune response. These cells reside mainly within a minor population of CD4 cells that express the phenotypic marker CD25 and FOXP3. They have been shown to suppress immunological responses against self [Sakaguchi, 2000] and foreign antigens [Suvas et al., 2003] through suppressive cytokines or direct cell–cell contact; however, regulatory effects of CD4+CD25+FoxP3 cells have not been fully elucidated [Maloy & Powrie, 2001]. It is possible that CD4+CD25+ T cells are responsible for the weak HBV-specific T-cell response in chronic hepatitis B patients and may inhibit the expansion and function of HBV-specific CD8 T cells, precluding HBV clearance but also limiting immune mediated liver damage.

The impact of circulating CD4+ CD25+ T cells on HBV pathogenesis has recently been analyzed. Increased frequencies of circulating regulatory cells in patients with chronic hepatitis B have been reported in some [Stoop et al., 2005] but not in other studies [Franzese et al., 2005].

Depletion of CD4+ CD25+ cells increased the function of HBV-specific T cells [Franzese et al., 2005; Stoop et al., 2005], but such modulation was not HBV-specific and could be observed in patients with resolved HBV infection [Franzese et al., 2005]. This casts doubts on the possible role of CD4+ CD25+ regulatory cells in the pathogenesis of chronic HBV infection. However, these studies were limited to the analysis of the CD4+ CD25+ cells present in the blood and a detailed analysis of the intrahepatic frequency and function of these cells is likely necessary to reveal their role. Furthermore, it is possible that a population of HBV-specific regulatory cells, different from the CD4+ CD25+ T-cell subset, analogous to the presence of IL-10 producing HCV-specific T cells [Accapezzato et al., 2004], might be induced in chronic HBV infection [Hyodo et al., 2004].

2.12 Neonatal Immune response

Deficiencies of T-Cell-Mediated Immunity in the Neonates

Neonatal life is characterized by heightened sensitivity to infectious agents. As during HIV infection, in the absence of retroviral therapy, there is a more rapid progression to disease in pediatric infection and this is associated with severely reduced HIV-specific cellular and humoral immune responses [Goulder, P. J., et.al, Br. Med. Bull. 2001, Luzuriaga, and K.et.al. AIDS Rev. 2002]. The sensitivity of newborns to infectious diseases might be partly due to the lack of pre-existing immunological memory in newborns. Another important contributing factor might be the small number of immune cells that are present in peripheral lymphoid tissues in early life, especially in mice. However, aside from these quantitative differences, many studies in both humans and mice have shown that newborn immune cells are qualitatively distinct from adult cells. Subsets of cells are present in different proportions in neonates and adults and, among cells

of the same subtype, phenotypic differences have been described [Adkins B. et.al 2004]. In addition, many in vivo and in vitro studies have described deficiencies or immune deviation among T cells, B cells and antigen-presenting cells (APCs) in neonates. Historically, the function of neonatal adaptive immune cells has been considered to be immature [Goulder P. J., et.al 2001]

Cytomegalovirus (CMV) is a ubiquitous herpes virus that ultimately infects 50–90% of the population. For the vast majority of children and adults, infection, which usually occurs after mucosal contact with bodily secretions, is either asymptomatic or results in a self-limited non-specific viral syndrome characterized by fever, hepatosplenomegaly, leukopenia, and myalgias [Gandhi and Khanna et.al 2004]. Cell-mediated immunity is essential for control of the disease, and onset of T-cell immunity results in resolution of viremia, although latent virus can be detected in tissues for life [Harari et.al 2004].

CMV Infection in utero may have dramatic, damaging effects on an otherwise healthy fetus [Brown and Abernathy, 1998; Gandhi and Khanna, 2004]. Although the majority of infected infants are asymptomatic, 5–10% will suffer severe neurologic damage including microcephaly, seizures, deafness, and retardation. Additional infants will appear asymptomatic at birth but will progress to have significant hearing loss. Infection acquired after birth is usually asymptomatic, but interestingly both congenital infection and post-natal infection result in prolonged shedding of the virus, while in adults such continuous shedding after primary CMV infection is limited to approximately 6 months after acquisition. This indicates an inability of the neonatal and infant immune system to control the virus compared to the immunocompetent adult [Stagno et.al, 1983].

A study by Tu et.al 2004 demonstrated that during CMV infection compared to adults, children had impaired virus specific Th1 responses but had relatively normal CD8+ T-cell responses

[Chen et al., 2004]. This was true even when primary infections of similar duration in both adults and children were compared. The delay in CD4+ T-cell immunity correlated with prolonged viral shedding in the urine [Tu et al., 2004]. Interestingly, CD8+ T-cell immunity seems to be relatively intact even in the setting of congenital CMV infection. Marchant et al. 2003 studied 8 newborns with congenital CMV infection compared to 15 healthy controls. Using tetramer staining and flow cytometry, they showed that CMV-specific CD8+ T cells could be detected pre-natally and early after birth, and these cells expressed IFN-γ, perforin, and granzyme A and could lyse target cells loaded with CMV peptides [Marchant et al., 2003]. Although this detailed study does not exclude a potential lag in the onset of CD8+ T-cell immunity in response to congenital CMV infection compared to postnatal infection, the robust nature of the immune response is very striking.

A robust CD8+ but not CD4+ T-cell response to congenital infection is not unique to CMV infection, as a similar pattern of immunity has been observed at birth in cases of congenital infection with Trypanosoma cruzi [Hermann et al., 2002]. This congenital infection results in a marked expansion of CD8+ T cells rather than CD4+ T cells, with evidence of oligoclonality of the TCR repertoire indicating that this is the result of antigen-driven expansion. These CD8+ T cells are enriched in markers for activation (HLA-DR high), memory (CD45ROhigh), and end-stage effector cells (CD28−/low), and for cytotoxicity (perforin+). They also have a markedly greater capacity to produce IFN-γ and TNF-α than CD8 T+ cells from uninfected newborns. In comparison, the CD4+ T cells in these congenitally infected newborns have undergone much less clonal expansion and acquisition of effector function [Hermann et al., 2002].

Newborns also are highly vulnerable to severe infection with herpes simplex virus (HSV)-1 and -2. Neonatal infection frequently results in death or severe neurological damage, even with the

administration of high doses of anti-viral agents, such as acyclovir [Kimberlin, 2004]. In contrast, death from disseminated primary HSV infection is distinctly unusual outside the newborn period, except in cases of T-cell immunodeficiency or in recipients of T-cell ablative chemotherapy or immunosuppression [Herget et al., 2005]. The increased disease severity in infants correlates with delayed and diminished appearance of HSV-specific Th1 responses, including CD4+ T-cell proliferation, IFN-γ and TNF-α secretion, and production of HSV-specific T-cell dependent antibody, compared to adults with primary infection Burchett. It is unknown whether CD8+ T-cell immunity is similarly diminished and delayed. It is also unclear by what age HSV specific CD4+ T-cell immunity achieves a level equivalent to that of adults.

Developmental limitations in immunity are not restricted to anti-viral immunity. Infections with Toxoplasma gondii, an obligate intracellular protozoan, are common with an overall seroprevalence of 22.5% in the USA [Montoya and Liesenfeld, 2004]. In adults, infections most often occur after ingestion of undercooked meat or food or water contaminated with cat feces, and are usually asymptomatic or result in mild, non-specific symptoms including non-tender lymphadenopathy. In immunocompetent adults, primary toxoplasmosis rarely disseminates to cause other sites of disease, such as chorioretinitis. Cell-mediated immunity driven by Th1 cells is required for containment of the infection, and deficiency in CD4+ T cells, IL-12, CD154, or IFN-γ results in increased severity of disease in animal models [Subauste et al., 1999]. In contrast to infection in adulthood, congenital infection, which occurs when the organism is transmitted transplacentaly to the fetus during an active maternal infection, can have direct consequences: While the majority of infants are asymptomatic at birth, a large number will progress to develop chorioretinitis and other neurologic complications.

Thus, neonates demonstrate striking deficiencies in cell-mediated immunity to a number of pathogens including viruses. A number of carefully orchestrated cellular and molecular events must occur before a strong cell-mediated immune response can be mounted, and newborns are transiently deficient at a number of points along the way. Neonatal antigen presenting function is also less efficient than that of adults, and, in particular neonatal dendritic cells secrete less IL-12. Neonatal CD4 cells are prevented from differentiating into Th1 cells due to decreased levels of STAT4 and epigenetic regulation of the IFN-γ promoter. They also express less CD154. Consequently, the normal positive feedback loops for driving cell-mediated immunity, in which IFN-γ and CD154 from T cells induce dendritic cells to produce more IL-12, are interrupted. Therefore, a better understanding of these cells and mechanisms will be important in the care of newborns as well as in pathogenesis and severity of diseases