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HUMAN IMMUNE RESPONSE TO ENVELOPE PROTEINS OF THE HEPATITIS B VIRUS

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Abbreviations

List of publications by the author

I. Samenvatting, Summary, Résumé

I.1. Samenvatting

Reeds meer dan 15 jaar wordt routinematig tegen hepatitis B virus (HBV) gevaccineerd. De actieve component van het vaccin is het manteleiwit van het virus, het hepatitis B oppervlakte antigen of HBsAg. Anti-lichamen gericht tegen het HBsAg-vaccin volstaan immers om de infectie die kan volgen op de blootstelling aan het HBV te voorkomen. Jammergenoeg blijken ongeveer 5 à 10% van de gezonde gevaccineerden niet in staat om een beschermende antilichaamtiter te produceren na een standaard hepatitis B immunisatie. Deze 'HBsAg nonresponders' zijn waarschijnlijk niet of onvoldoende beschermd. Het mechanisme verantwoordelijk voor deze gebrekkige immuunrespons tegen het HBsAg is niet bekend. Via een grondige analyse van de humane cellulaire reactie tegen HBsAg bij HBsAg-gevaccineerden wensen wij het mechanisme verantwoordelijk voor deze 'HBsAg nonresponsiveness' op te helderen. Deze kennis zou kunnen bijdragen tot de ontwikkeling van een nieuw, meer immunogeen vaccin dat bij iedere gevaccineerde een beschermende antilichaamtiter kan opwekken. Daar de afwezigheid van een immunologische reactie tegen de HBV manteleiwitten ook gezien wordt bij chronische dragers van het HBV, kan een grondige kennis van de humane immuunreacties tegenover het HBsAg ook heel nuttig zijn om ons te helpen begrijpen waarom chronische dragers er niet in slagen het virus te elimineren. Deze kennis kan, op langere termijn, eventueel leiden tot de ontwikkeling van een adequate therapie voor de behandeling van chronische infecties. Wij denken hierbij in de eerste plaats aan de ontwikkeling van een therapeutisch vaccin.

Deel 1: Relatie tussen HLA en 'nonresponsiveness'. Variaties in het immuunantwoord zijn vaak geassocieerd met polymorfismen van het MHC (Major Histocompatibility Complex). Het frequenter voorkomen van 'HBsAg-nonresponsiveness' bij personen met een HLA-*DR3* of -*DR7* haplotype suggereert dat deze nonrespons gestuurd wordt door genen van het MHC. In dit proefschrift worden HLA merkers van goede (*DR1,DR5,DP4,DQ3* en *DQ5*) en van zwakke (*DR7,DP11* en *DQ2*) respons geïdentificeerd. Vooral de combinaties *DR3~DR7* en *DP2~DR7* bleken frequent voor te komen in de nonresponder populatie.

Deel 2: Exploratie van de antigenpresentatie bij 'nonresponders'. Het immunologisch defect verantwoordelijk voor de 'HBsAg nonresponsiveness' kan op elk niveau van het humaan immuunantwoord liggen. In dit proefschrift wordt nagegaan of een defect in HBsAg-opname, -processing of -presentatie verantwoordelijk is voor de gebrekkige of afwezige anti-HBs productie bij nonresponders. Door middel van een functionele analyse toonden we aan dat het opname-, processing- en presentatiegebeuren correct verloopt bij deze personen.

Deel 3: Identificatie van HBsAg-specifieke T helper epitopen. Recent onderzoek toonde aan dat bij gevaccineerden de sterkte van de humorale immuunrespons op HBsAg grotendeels bepaald wordt door de kwaliteit van het T cel antwoord op dit antigen. Er werd immers een duidelijke correlatie gevonden tussen de in vivo anti-HBs productie en de in vitro T cel proliferatie. Om beter te begrijpen hoe HBsAg door T cellen wordt herkend, werden een aantal HBsAg-specifieke T cellijnen geproduceerd waarvan de epitoop-herkenning en HLA-restrictie werden bepaald. Bovendien werden, gebruik makend van lymfocyten uit het perifeer bloed van goede HBsAg-responders en van een reeks peptiden die 60% van de HBsAg sequentie omvatten, enkele dominante T cel epitopen van HBsAg geïdentificeerd.

Deel 4: Zoektocht naar verbeterde hepatitis B vaccins. Met het oog op de ontwikkeling van een meer immunogeen hepatitis B vaccin werden de veiligheid en de immunogeniciteit van een experimenteel hepatitis B vaccin (HBsAg met preS1 en preS2 sequenties) geanalyseerd in gezonde adolescenten en in nonresponders op het klassieke vaccin. Noch de gezonde adolescenten, noch de nonresponders op het klassieke vaccin reageerden beter op het experimenteel vaccin dan op het controle vaccin (Engerix-B®). Deze klinische evaluaties toonden aan dat de geselecteerde preS1 en preS2 gebieden de immunogeniciteit van het HBsAg-vaccin niet verbeterden. In een reeks in vitro experimenten werd aangetoond dat gedelipideerd HBsAg een verhoogde immunogeniciteit heeft vergeleken met het natief HBsAg. Ontvetting van HBsAg leidt tot een betere herkenning door T lymfocyten. De ontvetting resulteert evenwel in een minder goede herkenning van HBsAg door B lymfocyten en als gevolg daarvan in een geringere productie van anti-HBs.

I.2. Summary

Prevention of hepatitis B virus (HBV)-infection through vaccination has been routinely practiced for more than 15 years. The active compound of the vaccine is the envelope protein of HBV, the hepatitis B surface antigen or HBsAg. Antibodies to the HBsAg-vaccine have been shown to be effective in preventing infection after exposure to this agent. However, approximately 5 to 10% of healthy vaccine recipients fail to produce protective levels of antibodies to the hepatitis B vaccine after standard immunization. These HBsAg nonresponders may therefore be not or only inadequately protected. The mechanism responsible for this deficient immune response against HBsAg is unknown. By analyzing the human cellular reaction to HBsAg in HBsAg vaccinees we wish to unravel the mechanism responsible for HBsAg nonresponsiveness. This knowledge can be useful for the development of a new, more immunogenic vaccine that induces a protective anti-HBs response in all vaccinated individuals. Since a defective HBsAg responsiveness is also observed in chronic carriers of the HBV, new insights in human immune response to HBsAg, can help to understand why chronic carriers are unable to clear the virus. In the long run this knowledge may lead to the development of an adequate therapy to cure patients suffering from a chronic HBV infection. The development of a therapeutic vaccine may be one of the strategies to consider.

<u>Part 1: HLA and 'HBsAg-nonresponsiveness'.</u> Variations in the strength of the immune response are often associated with polymorphisms of the MHC (Major Histocompatibility Complex). The higher incidence of HBsAg-nonresponsiveness in individuals with a HLA-*DR3* or -*DR7* haplotype suggests that genes of the MHC are involved. In this thesis we identified HLA-markers of good (*DR1,DR5,DP4,DQ3* and *DQ5*) and of poor (*DR7,DP11* and *DQ2*) response. Especially the combinations *DR3~DR7* and *DP2~DR7* occur more frequently in the nonresponder population.

<u>Part 2: Immune defect responsible for 'HBsAg-nonresponsiveness'.</u> The immunological defect responsible for HBsAg nonresponsiveness may reside in each of the different steps of the human immune response. In this thesis we analysed whether a defect in HBsAg-uptake, -processing or -presentation is responsible for the inadequate or absent anti-HBs production in nonresponders. This functional analysis demonstrated that the processing and presentation phases are not deficient in these individuals.

<u>Part 3: Identification of HBsAg-derived T helper epitopes.</u> Recent research demonstrated that, in vaccinated individuals, the magnitude of the humoral immune response towards HBsAg is largely determined by the quality of the T cell response towards this antigen. A strong correlation between the in vivo anti-HBs production and the in vitro T cell proliferation was demonstrated. To better understand how T cells recognize the HBsAg, we generated a series of HBsAg-specific T cell lines and determined their epitope- and HLA-restriction. Furthermore, by using peripheral blood lymphocytes of good responders and a panel of overlapping synthetic peptides spanning 60% of the HBsAg sequence, we identified several dominant T cell epitopes.

<u>Part 4: In search of more immunogenic HBsAg-vaccines.</u> In the scope of the development of a more immunogenic hepatitis B vaccine, we analysed the safety and immunogenicity of an experimental hepatitis B vaccine (HBsAg with preS1 and preS2 sequences) in a cohort of healthy young adults and in nonresponders to the classic vaccine. These clinical evaluations demonstrated that the selected preS1 and preS2 regions do not improve the immunogenicity of the S-vaccine. Nor the healthy adults, nor the nonresponders to the classic vaccine reacted better to the experimental vaccine than to the control vaccine (Engerix-B®). A series of in vitro experiments revealed that delipidated HBsAg is more immunogenic as compared to the native HBsAg. The delipidated HBsAg is better recognized by T lymphocytes than the native HBsAg. However, delipidation of HBsAg reduced its B cell immunogenicity, and thus lowered the anti-HBs antibody production.

I.3. Résumé

La vaccination routinière contre l'hépatite B (HBV) se pratique depuis plus de 15 ans. Puisque les anticorps générés par l'antigène de surface de l'hépatite B (HBsAg) se sont montrés effectifs dans la prévention de l'infection après exposition au HBV, la partie active du vaccin contre l'hépatite B est composée de ces HBsAg (= des protéines de l'enveloppe du virus). Malheureusement, 5 à 10% des vaccinés en bonne santé sont incapables de produire des taux d'anticorps protectifs après une vaccination normale (standard). On assume que ces 'nonrépondeurs à l'HBsAg' ne sont probablement pas - ou seulement partiellement - protégés. Par le biais d'une étude approfondie de la réaction cellulaire humaine envers l'HBsAg auprès des personnes vaccinées à l'HBsAg, nous souhaitons éclaircir le mécanisme qui est responsable pour la 'nonréponse à l'HBsAg'. Cette connaissance pourrait être appliquée au développement d'un nouveau vaccin plus immunogène qui pourrait induire chez toutes les personnes vaccinées un taux d'anticorps protectifs. En outre on retrouve une même déficience de réponse immunologique chez les personnes atteintes d'une infection chronique d'hépatite B et par conséquent porteurs du virus de l'HBV. Une meilleure connaissance des réactions immunologiques humaines envers l'HBsAg pourrait nous aider à mieux comprendre pourquoi ces malades chroniques ne parviennent pas à éliminer le virus. Par conséquent on pourrait imaginer, dans le futur, de développer une thérapie adéquate, par exemple par le développement d'un vaccin thérapeutique.

1ère Partie: Relation entre 'HLA (human leucocyte antigen)' et nonréponse. Les variations en réponse immunologique sont souvent associées aux polymorfismes du CMH (Complex Majeur d'Histocompatibilité). La nonréponse à l'HBsAg se manifeste plus fréquemment chez des personnes portant l'haplotype HLA-*DR3* ou -*DR7*. Ceci suggère que cette nonréponse est dirigée par des gènes du CMH. Dans cette thèse nous avons pu identifier des marqueurs HLA (*DR1,DR5,DP4,DQ3* et *DQ5*) associés à une bonne réponse et des marqueurs HLA (*DR7,DP11* et *DQ2*) associés à une faible réponse. Nous démontrons qu'en particulier la combinaison *DR3-DR7* et la combinaison *DP2-DR7* se retrouvent plus fréquemment dans la population des nonrépondeurs.

2ème Partie: Exploration de la présentation de l'HBsAg chez les nonrépondeurs. La déficience immunologique responsable pour la nonréponse à l'HBsAg peut se situer à chaque niveau de la réponse immunologique humaine. Dans cette thèse, nous analysons l'hypothèse que chez les nonrépondeurs, un défaut à hauteur de la prise, de la décomposition ou de la présentation de l'HBsAg est responsable d'une absence ou d'un taux trop faible d'anticorps anti-HBs. Par analyse fonctionnelle nous démontrons que ces processus se déroulent d'une façon normale chez ces personnes.

3ème Partie: Identification d'épitopes T spécifiques envers l'HBsAg. Récemment il a été établi que l'ampleur de la réponse humorale envers HBsAg est en majeure partie déterminée par la qualité de la réponse des lymphocytes T envers cet antigène chez des personnes vaccinées. Il a été démontré qu'il existe une relation entre la production d'anticorps in vivo et la prolifération des lymphocytes T in vitro. Pour mieux comprendre comment les lymphocytes T reconnaissent l'HBsAg, nous avons généré une série de lignes de lymphocytes T spécifiques envers l'HBsAg. La reconnaissance de l'épitope et la réstriction HLA de ces lignes ont été déterminées. En plus, des épitopes T dominants ont été définis en utilisant des leucocytes provenant de bons répondeurs HBsAg et de peptides synthétiques couvrant 60% de la séquence HBsAg.

4ème Partie: Recherche d'un vaccin hépatite B amélioré. En vue du développement d'un vaccin plus immunogène contre l'hépatite B nous avons analysé la sécurité et l'immunogénicité d'un vaccin expérimental (HBsAg avec en addition des séquences du preS1 et du preS2). Ces études de vaccination ont été faites chez des adolescents en bonne santé et chez des nonrépondeurs envers le vaccin classique. Nous n'avons pas pu trouver une meilleure réponse envers le vaccin expérimental comparé au vaccin de contrôle classique (Engerix-B®). En conclusion, les évaluations cliniques ont démontré que les séquences sélectionnées (preS1 et preS2) n'amélioraient pas l'immunogénicité du vaccin HBsAg. Dans d'autres expériences in vitro nous avons démontré que les particules d'HBsAg délipidées ont un plus grand pouvoir immunogène que les particules natives. Une délipidation de l'HBsAg provoque une meilleure reconnaissance des lymphocytes T. Par contre, nous avons constaté une production moindre d'anticorps anti-HBs, ce qui nous permet de supposer que la délipidation de l'antigène cause une moindre reconnaissance par les lymphocytes B.

II. Introduction

II.1. General introduction

Hepatitis is a general term for an inflammation of the liver due to a variety of causes including metabolic diseases, drugs, alcohol, toxins, and viruses. Viruses are the most common causative agents of hepatitis today and infect many millions of individuals annually. Viral hepatitis encompasses several diseases and represents a global health problem. It induces major morbidity and mortality and places enormous demands on economic and medical resources. Several viruses that cause hepatitis have been isolated in the past decades: hepatitis A (formerly 'infectious' hepatitis), hepatitis B (formerly 'serum' hepatitis), hepatitis C (formerly 'non-A, non-B' hepatitis), hepatitis D (delta hepatitis), and hepatitis E (formerly 'enterically transmitted non-A, non-B' hepatitis). The 'hepatitis F virus', once classified as a hepatitis causing agent, was shown to be a mistake. Recently, a new virus (hepatitis G or GBV-C) has been identified as a sixth viral agent. It is possible that other viruses associated with hepatitis will be identified in the coming years [1,2].

The hepatitis B virus (HBV) is a major cause of infectious liver disease. Worldwide approximately 300 million people are persistent carriers of HBV. In East Asia and tropical Africa, where vertical transmission of HBV is the major cause of infection, more than 10 per cent of the population are chronic carriers (Figure 1). Chronically infected patients with active liver disease carry a high risk of developing cirrhosis and hepatocellular carcinoma (HCC). In fact, the risk of HCC is 100-fold in patients with chronic HBV infection [3]. The latency period between infection and cancer varies from 35 to 45 years and liver cancer is seen mostly in those parts of the world where childhood or perinatal infection is common. Most primary liver cancers (60% to 70%) globally occur in HBV carriers, and an estimated one million deaths from HBV related liver cancer occur annually. HBV-related primary hepatocellular carcinoma is one of the major causes of cancer-related death in major parts of Africa, Asia and Latin-America.

The clinical consequences of HBV infection are extremely variable and unpredictable. The virus can cause a disease of variable duration and severity, ranging from a subclinical form to acute hepatitis and severe chronic liver disease. After clinically apparent acute hepatitis B infection, approximately 90% of affected adults recover without sequelae, and develop lifelong immunity to the virus. In another 0.1-0.5% of cases a fulminant hepatitis results in which hepatocellular damage is so extensive that fewer than 50% of the patients survive. Finally, 5-10% of adults exposed to HBV are unable to eliminate the virus and develop a chronic infection. The nature of the chronic infection is also variable, ranging from continuous hepatocellular necrosis and inflammation (chronic active hepatitis) to an asymptomatic carrier state. In contrast to adult infection, neonatal HBV infection is rarely cleared and as many as 90% of perinatally infected children become chronically infected [4].

Until now, the mechanism of liver damage in HBV infection is not fully understood. Asymptomatic HBV carriers can have relatively normal liver morphology and function despite the presence of high levels of viral replication within their hepatocytes. This and a number of other observations have led investigators to the conclusion that liver injury and the subsequent clearance of virus during HBV infection is mediated by the

immune response, and that the HBV is not directly cytopathic [5]. Clearly, both arms of the immune response are involved in viral clearance. While the cellular immune response to the envelope, nucleocapsid, and polymerase antigens eliminates infected cells, the humoral antibody response to the hepatitis B surface antigen (HBsAg) contributes to the clearance and elimination of circulating virus particles after acute infection. This implies that an inadequate immune response leads to chronicity. Anti-HBs was clearly shown to be the serologic marker indicating the start of recovery in infected individuals and is the protective mechanism against re-infection in convalescent patients. Among other deficient immune responses, an inadequate cellular and humoral anti-HBs immune response has been observed in chronically infected patients, with no detectable anti-HBs in their serum. Immune unresponsiveness to HBsAg is thus considered to be a contributing factor in the pathogenesis of the persistent infection [6].

Since 1981 HBsAg-vaccination against HBV infections has been routinely practiced and antibodies to the HBsAg have been shown to be effective in preventing infection after exposure to this agent. Several studies have shown that these vaccines are safe, well-tolerated and effective. Since the current therapies for chronic HBV infection, such as IFNα, antiviral drugs and liver transplantation, are very expensive, have numerous and often serious side effects, and are, at best, only partially successful, active immunoprophylaxis (HB vaccination) seems to be the best way to cope with the problem of chronic hepatitis B. It is anticipated that effective HBV vaccine programs will interrupt the vicious cycle of perinatal infection, chronicity and late term complications including HCC. Until now, the increasing and systematic administration of vaccines slowly causes a decline in HBV incidence in both non-endemic and some endemic areas. Still, major impediments for the total eradication of HBV and its associated pathologies exist. Firstly, there is a non-negligible degree of nonresponsiveness following hepatitis B vaccination. Five to 10 percent of healthy young adults, 40% of hemodialysis patients, 25% of intravenous drug users and up to 90% of patients under immunosuppresive therapy display an inadequate antibody response (<10 IU/L) following a standard vaccination schedule with HBsAg vaccine. These non/poor-responders are not or inadequately protected against primary infection [7]. Secondly, there is the high frequency of chronicity following primary infection. These individuals are unable to clear the virus and form a permanent reservoir for the HBV.

Both groups, vaccinees as well as chronic carriers, are characterised by a deficient immune response to HBsAg. Until now, the mechanisms responsible for this HBsAg-nonresponsiveness, in vaccinees as well as in chronic carriers, remain largely unknown. However, the diversity of clinical syndromes induced by an HBV infection (acute versus chronic infection,...) and the variability of the humoral immune response to HBsAg after vaccination (high versus non/low responders) suggest a genetic regulation of the immune response to HBsAg in man, as was demonstrated in mice [8].

Whether the mechanism responsible for this immune deficiency is the same in both groups remains to be elucidated. In this thesis we tried to answer this question by unravelling the mechanism responsible for the immunological nonresponsiveness to HBsAg in vaccinees. By analyzing HBsAg-presentation, B7-

costimulation and HLA-linkage in hepatitis B vaccine nonresponders we were able to exclude some of the hypothetical mechanisms that could lead to this deficiency.

Since vaccination is the only route for eradication of HBV, development and delivery of a vaccine which is effective against all strains, and which induces a response in all vaccine recipients without exceptions, is an absolute requirement. The occurrence of nonresponsiveness and the reports of breakthrough infections following vaccination, due to the emergence of escape mutants with variant HBsAg sequences are signals to reconsider vaccine design [9]. By analyzing the genetic and cellular (especially the identification of dominant T cell epitopes) factors controlling the quality of the immune response to HBsAg and by modifying the HBsAg-vaccine, we hope to have contributed to the development of a more immunogenic HBsAg-vaccine.

The elucidation of the mechanism(s) responsible for the immunological nonresponsiveness to HBsAg and the development of a more immunogenic HBsAg-vaccine to induce an adequate immune response in all vaccine recipients, irrespective of the vaccination being prophylactic or therapeutic, are indispensible to combat HBV in the community and are, by consequence, the long-term objectives of this study.

II.2. Structural proteins of the hepatitis B virus

HBV is the prototype agent for a virus family called hepadnaviridae (hepatitis associated DNA viruses). All hepadnaviruses have a moderately narrow host range. HBV infects higher primates including men, chimpanzees and gibbons. Other monkeys are normally not infected. This limited host range of HBV and the lack of available in vivo and in vitro models, hindered the initial studies on HBV. The discovery of other hepadnaviruses has led to the development of alternative systems for studying the infection, replication and pathogenesis of HBV. Other members of the hepadnaviridae include WHV (woodchuck hepatitis virus), GSHV (ground squirrel hepatitis virus), TSHV (tree squirrel hepatitis virus), DHBV (duck hepatitis B virus), and the HHBV (heron hepatitis B virus). These viruses share the same structural and biologic characteristics, but have another host cell specificity. They all have small circular DNA molecules that are partly single stranded and an endogenous polymerase that repairs the DNA to make it fully double stranded. Species specificity (narrow host range), liver tropism (targetting of the virus to the hepatocytes), and the development of persistent infection further characterize this group. Although hepadnavirus infections have in the past been considered to be strictly hepatotropic, viral DNA, RNA and antigens have now also been detected in non-hepatocytes, including bile duct epithelial cells and endothelial cells in the liver, pancreas, kidney, adrenal cortex, skin, Kaposi sarcoma, spermatozoa, placenta, macrophages, spleen, bone marrow and a variety of peripheral white blood cells. It was demonstrated that once viral DNA has been internalized, most cell types are permissive, at least to some extent, for viral gene expression and DNA replication. Still, high-level viral gene expression and replication are only observed in the liver [11-14].

The hepatitis B genome (Figure 2) consists of a small, circular DNA molecule that is partially double-stranded. The DNA is composed of a long strand with a constant length (3182 bases) and a short strand which varies in length between 1700 and 2800 bases. The long strand carries virtually all the protein coding capacity of the virus and can therefore be considered as the minus strand. The short strand, devoid of coding function, is the plus strand. The circular structure of the DNA is maintained via baseparing at the 5' end of the 2 strands. This region contains identical direct repeat sequences of 11 to 12 nucleotides, designated DR1 and DR2. The direct repeat sequences are involved in the priming of plus and minus strand DNA, and the terminal redundancy is important for plus strand transfer and formation of the circular genome. In spite of their small genome size, hepadnaviruses are able to produce a remarkable variety of proteins. This is achieved through a combination of overlapping open reading frames (ORFs) and different start sites for translation to produce multiple related products from a single ORF with varying amino-terminal additions. The HBV genome contains four ORFs: the C-, the P-, the S-, and the X-ORF. The S region codes for the proteins of the viral envelope and is devided into the S gene, preS1 region and preS2 region. The C gene encodes the core protein and the P region encodes the viral DNA polymerase which possesses reverse transcriptase activity. The hepatitis B virus X protein (HBxAg) is a promiscuous transcriptional activator of polymerase II and III promotors, and up-regulates a wide range of cellular and viral genes. The function of HBx in viral replication and life cycle is still not completely elucidated.

The surface antigen ORF entirely overlaps with the P ORF [11,14-16].

Mature hepatitis B virions (Figure 3), also known as Dane particles, are surrounded by an outer membrane layer, the envelope, into which are inserted three related forms of a virally encoded transmembrane protein, the surface antigen. The lipids of this lipoprotein coat (fosfolipids, cholesterol and triglycerids) are derived from the host. The envelope encloses the nucleocapsid, an icosahedral structure formed by the viral core protein. The nucleocapsid contains the relaxed circular DNA genome, including the terminal protein covalently bound to the 5' end of the minus strand DNA, and the viral polymerase [17].

During HBV infection, at least four antigen-antibody systems are observed: hepatitis B surface antigen (HBsAg) and its antibody (anti-HBs); the preS antigens associated with HBsAg particles and their antibodies; the particulate nucleocapsid antigen (HBcAg) and anti-HBc; and an antigen structurally related to HBcAg, namely, HBeAg and its antibody (anti-HBe) (Figure 4). Antibodies are also produced to the non-structural polymerase [19,20] and X [21,22] proteins, primarily during chronic infections. The specific serologic marker of HBV infection is HBsAg, which is present both in the intact virion (42 nm diameter, infectious) and as excess free circulating filamentous (not infectious, different lengths, 22 nm diameter) and spherical 22 nm subviral particles (not infectious). These subviral particles can achieve concentrations approaching 100 μg/ml in the sera of chronic HBV carriers, and served as the source of the original plasma-derived HBsAg vaccines. Experience with HBV vaccination has taught that antibodies to HBsAg (major protein) are protective against HBV infections [23].

Three different forms of the HBsAg, termed the small (S), middle (M), and large (L) surface proteins (Figure 4-5). These are produced from a single ORF with three distinct translational start sites and one stopcodon. This explains why the 226 carboxyterminal AA of the three envelope proteins are identical. The smallest protein, called the major, S or small hepatitis B surface antigen (SHBsAg), is 226 AA long and is quantitatively the most important component in the empty virus particles as well as in the intact virions. The second envelope protein of HBV, called middle envelope protein (MHBsAg), is identical to the HBsAg, but has an amino-terminal extension of 55 AA, termed the preS2 sequence. The third envelope protein, called the large envelope protein (LHBsAg), is similar to the M protein, but is elongated at its amino-terminus with 120 AA, termed the preS1 sequence. The three envelope proteins can be glycosylated and, depending on the degree of glycosylation, SDS gel electrophoresis of purified particles shows three pairs of proteins or glycoproteins, respectively: P24 and GP27 for SHBsAg, GP33 and GP36 for MHBsAg, P39 and GP42 for LHBsAg. The larger protein of the pairs is the N-glycosylated form of the smaller one. There are two glycosylation sites, N146 of the S protein and N4 of the M protein. N4 is always glycosylated in secreted particles and N146 is only partially glycosylated when the S domain is part of the M and L proteins. S protein contains all the elements required for its own folding and can self-assemble, forming an aggregate of 60-120 transmembrane S monomers in host-derived lipids. These particles, produced either by recombinant means or purified from serum of infected subjects, are the basis of the current vaccines. It appears that a high proportion of LHBsAg is required for the maturation of HBV and for the

secretion of HBsAg filaments. The surface antigen proteins are cotranslationally inserted into the membrane of the ER. The envelope proteins have the ability to assemble into 22 nm spheres and filaments that bud into the lumen of the ER and the Golgi compartment. This auto-assembly process results in the production of a large excess of subviral particles compared to virions in the serum of viraemic individuals [24].

The spatial structure of the hepatitis B envelope proteins is incompletely understood but computer analysis of topogenic elements and mutational approaches have provided a model of the organization of the HBsAg protein [25,26](Figure 6a). Most current models for the transmembrane structure of S assume an external disposition of both the N- and C-termini, and four hydrophobic and two hydrophilic domains. Although four transmembrane α-helices are predicted, only the two most N-terminal domains are important for correctly anchoring the S protein to the membrane [27]. These two domains, corresponding to amino acids 11-28 and 80-98 respectively, are spaced by a hydrophilic region exposed on the internal side of the mature particle. This first hydrophilic region may thus be involved in attachment and development of the core particle during maturation of the virion. Deletion mutants reveal that this region tolerates only minor structural changes; three of the four cysteine residues in this region are conserved amongst all hepadnaviruses and were shown to be essential for secretion [28]. The second hydrophilic loop (amino acids 99-168) is exposed on the outer virion/particle surface since it contains the major group- and subtype-specific antigenic determinants and acquires carbohydrate modification. It is still unclear whether this region is directly involved in hepatocyte binding.

Receptor binding is probably a multi-step process involving several regions of HBV envelope proteins and including attachment of regions of the S protein, possibly to annexin V on the surface of the liver cell [29,30]. The external hydrophilic region has a highly complex structure and is very cysteine-dense: eight of the 14 cysteine residues in HBsAg are located here, and all eight are highly conserved among mammalian hepadnaviruses and HBV subtypes. There is, therefore, the potential for a high degree of secondary, tertiary and quaternary structure through the formation of intra- and inter-molecular disulphide bridges. Antigenicity is dependent upon this complex structure and substitution of many of the cysteine residues results in loss of immunoreactivity. However, not all cysteines are equally important for antigenicity and/or secretion [31]. The general role of cysteines in maintaining HBsAg conformation is well established, however, the exact structure of this region is unsolved. Structural predictions, use of cyclical peptides and phage library screening with monoclonal antibodies all provide stronger evidence for clusters of epitopes and a more complex structure [32]. The greatest affinity of anti-HBs is for epitopes in the second loop, antigenicity of which depends on key residues at AA 141-146 (KPTDGN). This has been studied using peptides in amino acid replacement assays [33] and by mutagenesis of yeast expressed HBsAg [34]. The major B cell epitope cluster of S protein is widely and historically referred to as the 'a' determinant. Many data have indicated that this spanned AA 124 to AA 147, but recent evidence suggests that this epitope cluster could be extended up- and downstream to include the entire major hydrophilic region (MHR). Although one would imagine the MHR to be a rigid structure, Wallace and Carman [9] hypothesize it to be a fluid structure where key antigenic regions are held in place by intra- and

inter-molecular bridging (Figure 6b). Some mAbs recognized epitopes that were up to 70 residues apart, which probably become spatially close when the molecule folds; alternatively, some epitopes may be contributed from adjacent S molecules. However, the exact fine structure of this region remains unresolved.

The hepatitis B virus surface protein has a degree of natural variation which is linked to ethnic and geographical backgrounds. This natural variability has evolved and become stable over the millenia. The serologic heterogeneity of the HBV has been established from immunodiffusion experiments for a long time. Four serotypes of HBsAg have been defined by two mutually exclusive determinant pairs, 'd/y' and 'w/r', and the common group-specific 'a' determinant. Therefore, the four subtypes of HBsAg (adw, ayw, adr, and ayr) represent the major viral phenotypes. By subdivision of the four major subtypes, nine different subtypes were identified. It was observed early that the ayw subtype was the one found among drug addicts worldwide, that the adw subtype dominated among the carriers in northwestern Europe, and that the subtypes with the 'r' determinant were exclusively confined to populations of the Far East [35].

A genetic classification based on the comparison of complete genomes has defined six genotypes of HBV, named A to F, defined by an 8% (up to 14% in genotype F) difference in sequence across the whole genome. Genotypes A to F are associated with distinct geographical areas. In addition there are nine subtypes, defined by a 4% difference in S gene sequence. By sequencing the S gene of HBV the molecular basis was assessed for the serological variations of HBsAg within the major four subtypes. However, the interrelation of the nine subtypes to the genotypes, the possible presence of more HBV genotypes as well as their global geographical prevalence remained to be determined. Variant HBsAg sequences have been detected worldwide and these appear to be antigenically relevant. There are two classes of variants: class 1 variants are found naturally and class 2 variants are observed in individuals that underwent a medical intervention (passive or active immunisation or antiviral therapy). Naturally occurring variants (reviewed in [36]) are usually not clinically significant, but there are examples which affect diagnostic assays. Class 2 variants, also referred to as mutants, are discussed in section II.5.8.

II.3. Hepatitis B virus and chronic hepatitis

HBV is a viral disease of global dimension with more than 300 million carriers of the virus worldwide. In countries where HBV carrier rates reach 10%, HBV infection may account for 3% of total mortality. The route of transmission varies geographically and is associated with the prevalence of HBV infection in this area. In areas of low HBV endemicity (Figure 7), infection occurs primarily in adolescents and adults and is transmitted sexually or parenterally. In areas of intermediate prevalence (carrier rate of 2-7%), HBV is spread horizontally, with the highest rate of infection occurring among older children, adolescents and adults. In areas with a high HBV prevalence (carrier rate of 8-20%), infection occurs primarily among infants and children through mother-child transmission (perinatal) and through close personal contact among children (horizontal). Although endemicity rates in Africa and south-east Asia are comparable, horizontal transmission predominates in Africa and vertical, perinatal transmission predominates in south-east Asia. The reason for this phenomenon is unknown, but this difference may in part be due to the higher prevalence of HBeAg in Asian (40%) than African (15%) mothers [38].

HBV chronic carriers represent a permanent viral reservoir, which hampers the total eradication of HBV and its associated pathologies. The diagnosis of chronic hepatitis B is established when transaminases (ALT/AST) are elevated and HBsAg is peristently present in serum for a period of at least six months. These patients are characterized serologically by the absence of anti-HBs, antibodies found in most individuals who have recovered from acute infection [39].

Chronic sequelae of HBV infection include chronic hepatitis (CH), liver cirrhosis and hepatocellular carcinoma (HCC)[40]. Epidemiologic studies and laboratory experiments showed that HBV is one of the most important etiologic factors of HCC, one of the most common cancers worldwide [41,42]. Primary hepatocellular carcinoma (PHC), induced by an HBV infection, is one of the most frequently encountered causes of death in major parts of Africa, Asia and South America [43,44]. CH has two forms: persistent (CPH) or active (CAH) depending upon the extent of portal triad disruption. In the Far East CAH is most often a mild or subclinical disease. Liver biopsy shows evidence of piecemeal necrosis with or without cirrhosis [45].

There is usually an inverse relationship between the levels of virus replication and liver inflammation. High levels of virus replication are associated with low inflammatory activity (CPH), reflecting the host tolerance and the immunological unresponsiveness to the virus. On the contrary, histological findings of CAH are usually associated with low levels of virus replication. The severity of the inflammatory changes reflects the intensity of the immunological response and the efficiency of virus clearance. These and other observations tend to indicate that chronic hepatitis B is not caused by a direct cytopathic effect of the HBV virus but by the host's immune response [5,46].

II.4. HBsAg recognition by the immune system

II.4.1. T cell recognition of antigen and MHC gene products: general properties.

An adequate immune response can only ensue when T lymphocytes recognize foreign antigenic fragments. T cells do not bind protein antigens directly, but recognize a heteromolecular complex consisting of a major histocompatibility complex (MHC) molecule and a peptide fragment of a protein antigen. The binding capability of the peptide-fragment to the MHC molecule depends on the primary sequence of the peptide and on allelic variation of the residues in the binding site of the MHC receptor [47,48]. HLA, the MHC of humans, is an assembly of closely linked genes of chromosome 6. The most remarkable characteristic of HLA genes (polygenic) is their genetic variability or polymorphism. Most of the genes are highly polymorphic, and many variants or alleles can be distinguished. The HLA gene products are codominant since each individual expresses two antigens per locus. An individual can be either homozygous (expression of 2 identical alleles) or heterozygous (expression of 2 different alleles) for a locus. Since HLA loci comprising the haplotype are closely linked, they are inherited as a genetic unit. Each individual, therefore, has two HLA haplotypes, one contributed by each parent. Based on their structure and function, HLA molecules can be divided in 2 major categories: 'HLA-class I' and 'HLA-class II' molecules (Figure 8). Both classes consist of cell surface glycoproteins. The HLA class I molecules, assigned as the HLA-A, HLA-B and HLA-C series, are present on the membranes of most nucleated cells. They are composed of a HLA-encoded α-chain with a MW of 45000, which is associated non-covalently, with a smaller beta chain of 11 kilodaltons. This smaller chain corresponds to the ß$_2$-microglobulin found in serum and urine and is encoded by chromosome 15. The heavy chain, which is very polymorphic, has three extracellular domains each consisting of about 90 AA. The HLA class II antigens are less polymorphic, have a more restricted distribution than the class I antigens, but have a more complex genetic and structural composition. They are mainly expressed on B cells, activated T cells, antigen-presenting cells (monocytes, macrophages and dendritic cells), and thymic epithelium. The three most important HLA class II molecules (HLA-DR, -DP, and -DQ) are heterodimers consisting of an α- and a ß-chain. Both HLA-encoded, non-covalently associated chains are inserted through the cell membrane via hydrophobic regions. The respective molecular weights are 33000 (heavy or α-chain) and 27000 (light or ß-chain). The HLA-DR locus can encode for one α- and three ß-chain genes. One gene coding for the DRα-chain, DRA, and one gene coding for the ß-chain, DRB1, is always present. Depending on the DR type, a DRB3, DRB4, or DRB5 gene may be present as well, eventually accompanied by pseudogenes. So, the HLA can express 1 or 2 HLA-DR molecules. The first DR molecule, encoded by the DRA (α-chain) and DRB1 (ß-chain) loci, is always expressed. The possible second DR molecule is encoded by the DRA and the DRB3, DRB4 or DRB5 loci. The DRB1 gene is the most polymorphic of the human class II genes with over 120 alleles identified, making it a powerful marker for individual identification. DRA is not polymorphic (only 2 alleles have been described) and some polymorphism is described in DRB3, DRB4, and DRB5. All these genes consist of 7 exons. Most of the allelic polymorphisms are located in exon 2, encoding the amino-terminal extracellular domain which functions as the antigen binding

site for processed proteins.

The HLA-DQ and HLA-DP loci each encode for two α- and two ß-chain genes. However, both the HLA-DQ and -DP regions only contain one functional gene each for the α- and the ß-chain. HLA-DQ molecules are encoded by the DQA1 and DQB1 loci. Both components are polymorphic, and both are thought to contribute to specificity. HLA-DP molecules are encoded by the DPA1 and DPB1 loci, both contributing to polymorphism [49].

The HLA alleles were designated by a consensus nomenclature adapted by the World Health Organization. Each year an update with newly assigned sequences, confirmations or corrections of previously reported sequences is published. Each HLA locus as it is defined, is assigned a letter in alphabetical order, e.g. HLA-A, HLA-B, etc. Universally accepted specificities are designated by Arabic numerals, such as HLA-A1, HLA-B5, HLA-DR3, etc. DRB1*XXXX refers to the genetic HLA typing. It was agreed that serologically identified products of e.g. the DRB1 locus would be known simply by the allele name, omitting the B1* (e.g. DRB1*0101 becomes DR0101). DR1, DR2,... refer to the associated serologic specificities [50].

The availability of the crystal structures of HLA molecules, initially reported for a HLA-class I molecule and later for class II molecules, has provided a breakthrough in the understanding of these molecules [51-53]. The basis of class I molecules is formed by ß2-microglobulin and the immunoglobulin-like α3 domain, while a distinct groove is formed by the α1 and α2 domain at the top of the HLA-molecule. This groove serves as a binding site for antigenic peptides for presentation to T cells. The 'floor' of the peptide binding cleft consists of ß-sheets, whereas the 'walls' are bound by extended regions of α-helical structure. The size of the antigenic peptides binding is generally 9 amino acids [54-57]. An analogous structure is found in HLA class II molecules, in that a peptide binding cleft sits on top of a base formed by the α2 and ß2 immunoglobulin-like domains (Figure 9). Moreover, the geometry of the cleft itself is highly similar, but not identical, to that found in the class I molecules. The major consequences of the differences in structure between HLA class I and II antigenic peptide binding grooves is that class II molecules can bind peptides that are longer and more variable in size; namely from 12 to 19 amino acids [51,57].

A fundamental difference between class I and class II molecules, besides the different size of peptides bound in the cleft, is the source of these peptide antigens. Generally, class I molecules present peptide antigens derived from proteins that are actively synthesized within the endoplasmatic reticulum, whereas class II molecules present antigens taken up from outside the cell by endocytosis in vesicles called endosomes. These differences between class I and class II molecules are reflected by two distinct pathways of antigen processing and presentation: the exogenous or MHC class II-restricted pathway and the endogenous or MHC class I-restricted pathway (Figure 10). The exogenous pathway operates e.g. during immunization with soluble protein antigens and stimulates a CD4^{+} T cell response. Human CD4^{+} T cells recognize short antigenic peptides present in the antigen-binding groove of HLA class II molecules at the surface of APC such as dendritic cells, macrophages and B cells. The antigenic peptides are derived by proteolytic cleavage of exogenous antigen that has been

phagocytosed or endocytosed by the APC and delivered into the lysosomal pathway where the derivative peptides associate with HLA class II molecules and are delivered to the cell surface for recognition by the appropriately rearranged T cell receptor (TCR) on a CD4$^+$ T cell. The T cell subset specificity of this interaction derives from the fact that the multimolecular HLA-peptide-TCR complex is stabilized by accessory interactions such as those between the CD4 molecule on the T cell and the HLA class II molecule on the APC [reviewed in 58,59]. A cytotoxic T lymphocyte response is stimulated by protein antigens processed in the alternative endogenous pathway. Human CD8$^+$ T cells recognize short antigenic peptides (usually 9-11 residues) present in the antigen-binding groove of HLA class I molecules present at the surface of the cells. The antigenic peptides are derived by proteolytic cleavage in the proteasome of endogenously synthesized proteins in the cytoplasm. These may be self proteins, microbial- or tumor-derived molecules. The processed peptides are then bound by a family of transporter proteins (encoded within the HLA locus) which shuttle them into the lumen of the ER where they are scanned for the presence of HLA-allele-specific binding motifs by the antigen-binding domain of resident HLA class I proteins. Peptides containing the appropriate motif are tightly bound by the corresponding HLA class I molecule which then associates with ß2-microglobulin and moves to the cell surface as an integral membrane protein where it can present the antigenic peptide to the T cell receptor on a CD8$^+$ T cell. The T cell subset specificity of this interaction derives from the fact that the multimolecular HLA-peptide-TCR complex is stabilized by accessory interactions such as those between the CD8 molecule on the T cell and the HLA class I molecule involved in the complex [reviewed in 60]. Although there is a small amount of cross-talk between these two pathways, the CD4$^+$ T cell response is principally class II restricted and regulatory in its function by secreting lymphokines which modulate the activity of antigen-specific B cells and CD8+ T cells as well as other CD4$^+$ cells and macrophages. By the same token, the CD8$^+$ T cell response is principally class I restricted and functions as the antigen-specific effector limb of the immune response as a CTL.

It is now accepted that costimulatory interactions are necessary for effective lymphocyte activation and also serve to enhance the immune response (Figure 11). Both the engagement of the TCR with MHC/Ag (first signal) and a second costimulatory signal are needed for the complete activation of the T cell. The CD28/B7 receptor/ligand system is one of the dominant costimulatory pathways. CD28 interactions with the B7 family of costimulatory ligands are essential for initiating antigen-specific T cell responses, upregulating cytokine expression and promoting T cell expansion and differentiation. Interruption of this signaling pathway with CD28 antagonists not only results in the suppression of the immune response, but in some cases induces antigen-specific tolerance. However, the CD28/B7 system is increasingly complex due to the identification of multiple receptors and ligands with positive and negative signaling activities [61,62].

II.4.2. Human humoral immune response to HBV envelope proteins
Serologic studies in HBV infection have clearly shown that structural and non-structural proteins are variably immunogenic at the B cell level. The HBsAg protein of the viral and subviral particles, although significantly

less immunogenic than HBcAg, displays the major B cell antigenic determinants that can induce a protective immune response. Serologically, HBsAg contains a common epitope, referred to as the group-specific 'a' determinant, and two sets of mutually-exclusive subtype determinants, 'd' or 'y' and 'w' or 'r'. The combination of the common (group) and subtype determinants results in the four major subtypes of HBsAg: adw, ayw, adr and ayr. In general, the anti-HBs immune response in humans mainly targets the 'a' determinant which is associated with all subtypes of HBV. Since recovery from infection by one subtype confers immunity to the others, and immunisation with one HBsAg subtype confers protection against HBV infection with all subtypes, it may be concluded that anti-a antibodies are protective [63]. It has been shown previously that the common 'a' determinant is located between AA 124 and AA 147 [64]. Recent evidence however suggests that this epitope cluster could be extended up- and downstream to include the entire major hydrophilic region [9]. Studies suggest that the 'a' determinant contains several non-overlapping epitopes (Table 1), indicating that it is not a single determinant, but rather a conformation of several epitopes located on different regions of HBsAg. Since the exact disulfide linkage data are lacking, the tertiary structure of the 'a' determinant remains undefined. Variations at HBsAg-sites 122 and 160 alone define the d/y and w/r variations, respectively. More details about the group and subgroup determinants are described in [36]. The antibody response to HBV envelope antigens is strictly T cell-dependent, i.e. production of anti-envelope antibodies by immunization with the corresponding envelope proteins requires the presence of specific Th lymphocytes capable of recognizing and responding to T cell epitopes within the envelope region. The appearance of anti-envelope antibodies in a naturally infected subject indicates viral elimination.

Several groups have initiated studies to determine the occurrence and kinetics of antibody production to the preS1 region during acute and chronic HBV infection. It was found that antibodies to preS1 appear at the early stage of acute resolving HBV infection, but the antibodies are absent or of differing specificity in the sera of patients with acute HBV infection evolving towards chronicity and in the sera of chronic carriers [65,66]. In view of the early appearance of anti-preS1 in acute infection and its temporal relationship with clinical recovery, Alberti et al.[65] and Neurath et al.[67] analysed in detail the fine specificity of anti-preS1 antibodies. It was demonstrated that the preS1 sequence 12-32 is recognized by antibodies in sera of individuals recovering from HBV infection. It was noted that anti-(12-32) can be detected prior to anti-HBs or anti-HBc seroconversion but declines rapidly after production of anti-HBs and anti-HBc. The human anti-preS1 response can include multiple specificities since peptide sequences represented by 1-21, 12-32, 32-53, and 94-117 were variably recognized by sera from different HBV-infected patients. B-cell epitope 1-21 is unique to human sera and epitope 41-53 is unique to murine sera. The remainder of the B-cell sites are shared by mice and man. In a study of chronically infected patients [68], an interesting observation was that among asymptomatic carriers anti-preS1 was detected in those seropositive for anti-HBe but not in those positive for HBeAg. The authors suggested that anti-preS1 may have a role in clearing HBV from the circulation because preS1-containing polypeptides are preferentially expressed on virions.

Several groups have examined in vivo antibody production to the preS2 region of HBsAg during HBV infection. Using a synthetic peptide representing residues 120-145 within the preS2 region, it was demonstrated that HBV-infected acute phase sera contain antibodies that recognize this sequence [67]. Examination of the fine specificity of antibodies reacting with the 120-145 sequence revealed that multiple specificities exist that primarily recognize the 133-143 epitope, which is also recognized by murine sera. The preS2-specific antibodies appear early during infection and decline in titer rather rapidly. Several studies have suggested a transient nature for antibodies to preS2 during HBV infection. No anti-preS2 was detected in acute patients who progressed to chronicity or in patients with chronic hepatitis with persistence of HBsAg and preS antigens regardless of HBeAg/anti-HBe status [69].

II.4.3. Human cellular immune response to HBV envelope proteins.

Sensitization of CD4$^+$ lymphocytes to HBsAg proteins can rarely be demonstrated in HBV infected patients [70-74]. This may be explained by the very low frequency or absence of HBsAg-specific T cells but can also relate to the low immunogenicity of HBsAg (100-fold less compared with HBcAg in mice) and the lack of sensitivity of the cellular assays. When the T cell response to the envelope antigens is serially studied during acute infection it is weak. In contrast, the envelope-specific T cell response is usually quite strong in a good proportion of vaccine recipients who have been immunized with plasma-derived or recombinant HBsAg. Recognition of surface proteins by human T lymphocytes and their fine specificity has been studied in vaccine recipients as well as in patients suffering from acute and chronic infections (Table 1). Celis et al.[75] first described HBsAg-specific CD4$^+$ T lymphocytes derived from peripheral blood of vaccine recipients. These T cell clones proliferated and produced IFNγ upon stimulation with HBsAg. They also supported anti-HBs production by autologous B lymphocytes in the presence of HBsAg. Another study of the same group mapped a dominant HBsAg-specific T cell recognition site to residues 193-202 (19-28) within the N-terminus of the S-region. This immunodominant epitope is recognised in association with HLA-class II DPw4 molecules ('restriction determinant') [76,77]. The majority of the HBsAg specific T cells exhibited both proliferative and cytotoxic responses, although they were phenotyped as being CD4$^+$. Barnaba et al.[78] revealed that the overlapping region 193-207, was recognized in a DR2(15) context. Results from Penna et al.[79] indicate that HBV envelope-specific, HLA-class II restricted CD4$^+$ CTL can potentially participate in the immune clearance of HBV-infected cells and the pathogenesis of hepatocellular injury in hepatitis B. Rao and coworkers demonstrated that peptide 124-147 of the S region encodes a dominant, conformational, group-specific epitope that is recognized by human anti-HBs Ab [80]. Further experiments revealed that this peptide also contains at least two T helper epitopes, one located between residues 124-137 and the other between residues 139-147 [81]. This was consistent with previous results from other laboratories [82]. Recently, Min et al.[83], Deulofeut et al.[84], and Honorati et al.[85] defined one and three new epitopes in HBsAg, respectively.

In another study, peripheral blood lymphocytes of 14 vaccine (plasma-derived) recipients were examined for

envelope-specific T cell activation [86]. All vaccine recipients displayed a significant proliferative T cell response to the S protein, eight subjects responded to the preS1 region product and only one person responded to the preS2 antigen. Limiting dilution studies in 2 vaccinated individuals showed that the precursor frequency of preS1-specific T cells was higher than that of S-specific T cells. Furthermore, preS1-specific T cell lines, besides their HBsAg-specific proliferation, show also HBsAg-specific HLA-class II restricted cytotoxic activity. It was concluded that the preS1-region is a strong immunogenic region in men. Unfortunately, the impact of the HBsAg subtype was not analysed in this study which could explain the low preS2 specific response. Subsequent studies, conducted by the same group and based on antigen recognition by T cell clones, mapped immunogenic epitopes within the preS1 protein; AA 21-30 and AA 29-48 [87]. Further experiments by Serra et al.[88] revealed that PBMC expressing DQ1(5) and DQ1(6) subtypes were very efficient in presenting peptide 21-30. A CD8^{+}, MHC-class I restricted (HLA-A11) human T cell clone has also been derived from a vaccine recipient. The epitope recognized by the CD8^{+} clone was mapped to residues 21-28 within the preS1 region [89]. Using Vaccinia/HBsAg recombinant vectors, these authors could demonstrate that class I- as well as class II-restricted T cell clones could recognise an endogenous expressed as well as an exogenous administered antigen. These results suggest that a subunit vaccine can induce class I-restricted T cells that are able to recognize virus infected cells.

Cupps et al.[90] studied the immunogenicity of the preS2 protein in vaccinated individuals and identified a number of serotype-specific T cel epitopes in this region. The most important epitope for T cell proliferation was localised in the preS2 (AA 146-165) region (adw subtype). Cross-reactivity with ayw serotype-specific peptides not included in the preS2-S vaccine, was limited. This observation can be useful for further vaccine development. Steward et al.[91] identified the AA 126-140 within the preS2 region as a T epitope. Barnaba et al.[92,93] have examined T cell sensitization to HBsAg proteins in patients suffering from chronic HBV infections. MHC class I- and class II- restricted T cell clones were generated from liver infiltrating lymphocytes that were stimulated in vitro with preS2 containing HBsAg particles. These clones recognized determinants within the preS2 residues 120-134, suggesting that this sequence can bind to MHC class I and MHC class II molecules. Specific cytotoxic responses against HLA-matched EBV-immortalized lines sensitised with HBsAg particles or peptide 120-134 were displayed by both types of clones. This indicates that preS2 specific T cells are present within the liver during chronic infection and that these cells may mediate hepatocellular lysis. These investigators have also suggested a mechanism whereby pre-S2-specific cytotoxic T lymphocytes may lyse HBsAg-specific B cells that present envelope peptides in the context of the MHC-class I pathway [93]. Such a mechanism would result in the suppression of anti-HBs production, observed during chronic infection.

Anti-viral CTL are believed to play a major role in eradication of infection by virtue of their capacity to identify and kill virus-infected cells through recognition of viral peptides presented by HLA class I molecules. Due to the restricted host-range of HBV and its lack of infectivity in vitro, the CTL response to this virus has been very difficult to study. Thanks to the analysis of MHC bound peptides, a procedure pioneered by

Rammensee and coworkers [94], it has become possible to define the epitopes within the envelope protein recognised by human CD8+ lymphocytes during acute hepatitis B. By using a strategy involving in vitro stimulation of PBMC with HBV-derived peptides, selected according to the HLA-A2 binding motif as described by Rammensee's group, Nayersina et al.[95] showed that most patients with acute viral hepatitis produce a CTL response to several HLA-A2 restricted epitopes. They demonstrated that the CTL response in acutely infected patients is polyclonal and multispecific within the individual patient and is absent in uninfected healthy volunteers and chronically infected patients. For the first time these data confirm directly the involvement of virus-specific CTL in host defence during acute hepatitis B virus infection and support the hypothesis that viral persistence may be related to a low level CTL response in chronically infected patients. Only recently, Rehermann et al.[96] were able to demonstrate that chronically infected patients who experience a spontaneous or interferon-induced remission develop a CTL response to HBV that is similar in strength and specificity to patients who have recovered from acute hepatitis. The HLA-A2 restricted envelope-specific CTLs recognizing amino acid residues 194-202 and 346-354 were identified. These results suggest that specific immunotherapeutic enhancement of the CTL response to HBV should be possible in chronically infected patients, and that it could lead to viral clearance in these individuals.

It is widely believed that the HBV is completely cleared by antiviral antibodies and specific CTLs during acute viral hepatitis. However, it was demonstrated recently that traces of HBV are often detectable in the blood for many years after clinical recovery from acute hepatitis, despite the presence of serum antibodies and HBV-specific-CTLs, which can be present at acute-stage levels. These results suggest that complete clearance of HBV frequently fails to occur after recovery from acute hepatitis and that traces of virus are kept under the control of a vigilant immune system for decades following clinical recovery [97]. When the immune system is severely depressed (endstage renal disease, HIV infection, immunosuppressive therapy,..) reactivation of the HBV remnant may occur.

II.5. Current data on hepatitis B vaccination

II.5.1. General

The single most important tool for the prevention and control of hepatitis B infections is the hepatitis B vaccine. The first experiments on active immunisation against hepatitis B were reported by Krugman et al.[23]. Nowadays, active immunisation against HBV infection is common practice. Since antibodies to the HBsAg have been shown to be effective in preventing infection after exposure to this agent, the development of an efficient vaccine has slowed down the steady increase of chronic HBV hepatitis, liver cirrhosis and HCC. Systematic vaccination of neonates born from HBV chronic carrier mothers prevents the development of chronic carriership in these children and so disrupts the vicious circle. To date, the rationale for universal immunisation of neonates against HBV in countries with high or intermediate endemicity of HBV is widely accepted [126]. Indeed, over 40 countries are in the process of introducing universal HBV immunisation into the activities of their EPI program (Expanded Programme on Immunisation), or have already done so (Figure 12)[127,128]. The EPI is a multinational effort to immunise all of the world's children against the immunisable diseases of childhood. If consistently applied, this policy may lead to elimination of hepatitis B as a serious disease of humankind by the year 2050 (Figure 13). Cost is the major limiting factor affecting implementation of universal immunisation to neonates in Africa and most Asian countries. Data obtained through cost-benefit analysis in the past decade repeatedly suggest a long-term benefit of such an immunisation policy [130]. However, many countries are unable to allocate the short-term funds necessary to introduce an effective prevention program against HBV, even though the long-term cost-benefit analysis justifies such an effort.

The 'first generation' hepatitis B vaccines were obtained by purification and heat-inactivation of HBsAg particles that were derived from the plasma of chronic HBV carriers [63,131,132]. The first plasma-derived hepatitis B vaccines (PDV) were licenced in France and the U.S.[63] in 1980 and became available since 1982. These vaccines were the first such products to be manufactured from human plasma and possessed the additional attribute of being the first anti-cancer vaccines to become available for human use. Their safety and immunogenicity was demonstrated in many studies with healthy individuals and with persons belonging to higher risk groups (homosexual men [63,133], health care workers [134], haemodialysis patients [135,136],..). The high cost-price and the limited supply of plasma of chronically infected persons are disadvantages of these vaccines. Furthermore, the fear, although groundless, concerning the biological safety of these products, has hampered the general acceptance of this vaccine. The recombinant DNA technology induced the development of 'second generation' hepatitis B vaccines. The HBsAg, present in these vaccines, is produced by recombinant yeast or mammalian cells wherein the HBsAg gene (S gene) is cloned [137-139]. These recombinant, yeast-derived vaccines (YDV) became commercially available in 1986 and are now in use worldwide [140]. Both plasma and yeast derived vaccines have had an excellent safety record. Large scale production of non-glyco-sylated HBsAg in yeasts is already cost-effective, and costs are declining rapidly. The available HBV vaccines, whether PDV or YDV, are highly immunogenic, leading to 90 to 96% seroconversion rates in pediatric and

young adult populations [141].

Although currently available HB vaccines are very immunogenic, not all vaccinees are able to mount a protective antibody titer after a correct administration of 3 or more vaccine doses. Approximately 1 to 3% of neoborns and up to 6% of immunocompetent adults produce no or inadequate (<10 IU/l) anti-HBs levels after vaccination [7]. Since little is known about the T cell priming effect of an unsuccessful vaccination, nonresponders should be considered to run the same risk for HBV infection as unvaccinated subjects. Little is known about the course of an acute HBV infection in nonresponders, but the few reports available suggest that the severity and evolution towards chronicity in vaccine nonresponders is not different from that observed in unvaccinated subjects [142-144]. Furthermore, there are a number of target populations which do not readily seroconvert when immunised with the conventional HBV vaccines. These groups include older adults and therapy- or disease-related immunodeficient patients (e.g. chronic dialysis patients, HIV-infected individuals,...)[145]. Chronic carriers of HBV (healthy, asymptomatic carriers as well as chronic active hepatitis patients) are also characterized by a deficient humoral response to HBsAg [146]. Since the appearance of anti-HBs predicts recovery in acutely infected persons, clinicians hope to overcome chronicity in these individuals by therapeutic vaccination with HBsAg [147,148]. Unfortunately, the results of such therapeutic vaccination trials have been deceiving.

The presence of nonresponders to the available hepatitis B vaccines, and the finding that the strength of the initial immune response to the vaccine is an important contributing factor in the duration of the protection [149], argued in favour of the development of a more immunogenic vaccine that induces a protective antibody titer in all vaccinees. The research in HB vaccinology is concentrated mainly on the development of this 'third generation' vaccines.

II.5.2. Immune response to the HB vaccine

More than a decade of experience in hepatitis B vaccination has shown that the immune response to the hepatitis B vaccine in healthy subjects is extremely variable. Following administration of 3 doses of vaccine under optimal conditions, most recipients produce a high-titered and long-lasting anti-HBs response. However, 5 to 10% of healthy vaccinees respond slowly and poorly (anti-HBs titer ≤ 100 IU/l) and maintain protective antibody levels (≥ 10 IU/l) for only very limited periods. Approximately 1 to 3% of neonates and up to 10% of healthy adults do not produce adequate levels (<10 IU/l) of antibodies after 3 doses of the currently available vaccines, and can be considered nonresponders [150]. This nonresponsiveness is a selective phenomenon and is not the expression of a general immune deficiency. This update will concentrate on the HB vaccine nonresponsiveness as observed in healthy individuals.

The immune response to the hepatitis B vaccine is determined by numerous immunisation and host factors. **Immunisation factors** include the dose of vaccine administered, the vaccination schedule, the site and route of immunisation, and the storage conditions of the vaccine. Numerous clinical trials and more than 10 years of

experience in the field have shown that optimal results are achieved when 3 or 4 doses of 20 μg of HBsAg vaccine are administered intramuscularly in the deltoid region at 0, 1 and 6 months or at 0, 1, 2 and 12 months, respectively [151]. **Host-related factors** which influence the response to hepatitis vaccination include age, gender, bodyweight, smoking habits and disease state. The immune response to hepatitis B vaccine, expressed in both seroconversion rate and the geometric mean anti-HBs titer, declines with increasing age [141,152,153]. Obesity and smoking also have an adverse effect on the anti-HBs response [154-157]. In general, women produce a better immune response than men, and this may be related to hormonal and postural or bodyweight differences between both sexes [158]. Moreover, conditions which impair the function of the immune system may hamper the response to the vaccine. This has been observed in homosexual men infected with the human immunodeficiency virus (HIV) [159-161] and in intravenous drug users [162]. Approximately 40% of haemo-dialysis patients [163-165] and 70 to 80% of organ transplant recipients receiving immunosuppressive therapy [166,167] cannot mount adequate anti-HBs responses. Apart from the usual host-related factors which account for this variability in immune response, a genetic factor is recognised as a possible cause of nonresponse.

II.5.3. Genetic control of the immune response to the HB vaccine: MHC class II association

II.5.3.1. Genetic control of the murine immune response to HBsAg

Through the work of Milich et al. much is known about the regulation of the immune response to HBsAg in a murine model [8,98,100,109,168-170]. The most important findings are the following:

a) the humoral immune response of different inbred mouse strains to HBsAg is highly variable.

b) The antibody production after immunisation of mice with HBsAg is controlled by the MHC-system. A marked influence of the H-2 linked immune response genes on the kinetics and magnitude of the anti-HBs production was shown. High responder ($H-2^{d,q}$), intermediate to low responder ($H-2^{a,b,k}$), and nonresponder ($H-2^{s,f}$) haplotypes can be identified [8,168,169].

c) The production of preS2-specific antibodies, generated after immunisation with preS2-S or preS1-preS2-S particles, is also controlled by the H-2 system.

d) The genetic regulation of the immune response to preS2 and preS1 antigens is independent of the immune response to the S antigen. After immunisation with preS2-S particles of $H-2^s$ mice (nonresponder to S, but responder to preS2), S-specific antibodies were produced besides preS2-specific antibodies [170]. After immu-nisation with preS1-preS2-S particles of $H-2^f$ mice (nonresponder to S and preS2, but responder to preS1) S and preS2-specific antibodies were produced besides preS1-specific antibodies [109]. Analysis of the S- and preS-specific T responses revealed that the mechanism that permits circumvention of this nonresponsiveness, can be situated at the T cell level. The ability to induce a S- and preS-specific response in non-responder mouse strains after immunization with preS1-preS2-S particles suggests that these mice have preS1-specific T cells who can help not only preS1-specific, but also S- and preS2-specific B cells [98].

e) The humoral immune response to the group-specific 'a' and the subtype-specific 'd' and 'y' determinants of

HBsAg is controlled by H-2 linked immune response genes [100].

f) Responsiveness to HBsAg is inherited as a dominant trait, whereas nonresponsiveness is inherited as a recessive trait.

g) no evidence for immune suppression was found in nonresponder mice.

II.5.3.2. Genetic control of the human immune response to HBsAg

The suggestion that genetic mechanisms may control the human immune response to HBsAg was made by different authors who found a statistically significant association between certain HLA-haplotypes and nonresponsiveness to the HB vaccine (Table 2). In the literature we will find two opposit opinions concerning this nonresponsiveness. Both theses will be discussed in detail and can be summarized as follows: 1) a normal response to HBsAg is due to the presence of one or more dominant immune response gen(es) positioned in the HLA-region; poor or nonresponsiveness on the other hand is caused by the absence of these gene(s) and the presence on both chromosomes of HLA-haplotypes inducing a nonresponse. In this vision, the lack of a normal response was a recessive MHC-linked trait [171-176]; 2) the absence of a humoral immune response to HBsAg is caused by the presence of an immune suppression gene located in the HLA-region. Nonresponse is then inherited as a dominant trait and is mediated by CD8^{+}, HBsAg-specific suppressor T lymphocytes [177,178].

The suggestion that MHC-linked genes may control the human immune response to HBsAg was first made in 1981 by Walker et al.[171]. A significant excess of HLA-DR7 and a total absence of HLA-DR1 was observed in a group of 23 subjects showing a very weak or absent anti-HBs response to 3 doses of vaccine. This observation was later confirmed and extended by other investigators. In hypo- and nonresponders to a first series of 3 hepatitis B vaccines, HLA-DR7 [172,173] and HLA-DR3 [173,174] were found more frequently than in the general population. A greater than expected number of homozygotes for the extended MHC haplotype (HLA-B8/SCO1/DR3) was observed in low/nonresponders, which suggested that the lack of a normal response was a recessive MHC-linked trait. This hypothesis was subsequently confirmed in a study wherein 5 homozygotes and 9 heterozygotes for the extended haplotype (HLA-B8/SCO1/DR3) were prospectively vaccinated [176]. Four of the 5 homozygotes produced low levels (<1000 RIA units) of anti-HBs, whereas all 9 heterozygotes produced normal levels of antibody. It was concluded that a normal humoral response to HBsAg was governed by a dominant immune response gene located in the MHC, and that low or nonresponsiveness was caused by the absence of this gene and the presence on both chromosomes of a recessive inherited MHC-linked non-response gene(s) for HBsAg. Recently, it was shown that the genes encoding the HLA-DQ2 are subtyped into DQB1*0201 (in linkage disequilibrium with DR3) and DQB1*0202 (in linkage disequilibrium with DR7). The lack of antibody response to HBsAg vaccination was shown to be significantly associated with the HLA haplotype DRB1*0701-DQB1*0202. A second HLA haplotype, DRB1*0701-DQB1*0303, on the other hand, was not associated with the lack of antibody response to HBsAg vaccination, indicating a crucial role for the DQB1*0202 allele [179].

A different hypothesis on the mechanism of nonresponsiveness to HBsAg has been put forward by Sasazuki's group [175,177,178]. These investigators immunised 85 healthy Japanese volunteers via the subcutaneous route with 3 doses of a plasma-derived vaccine. Four weeks after the third dose, 19 subjects (22,4%) showed no anti-HBs response. Among the nonresponders there was a significant increase of the HLA-Bw54/DR4/DRw53 haplotype. This research group further showed that responsiveness to HBsAg was a recessive trait, whereas nonresponsiveness was transmitted dominantly and was mediated by $CD8^+$, HBsAg-specific suppressor T lymphocytes. The increased incidence of the HLA-Bw54/DR4/ DRw53 haplotype in nonresponders, as well as the dominant inheritance pattern of this nonresponsiveness and the role of suppressor T cells in its expression are observations made only by Sasazuki's group [177]. These unique results may be related to the oriental origin of the subjects studied and/or the subcutaneous vaccination route used.

Although HLA-identical siblings generally produce virtually identical levels of anti-HBs, the dramatic difference in antibody responses observed in one pair [180] suggests that unidentified, non-MHC-linked factors may influence the anti-HBs response.

II.5.4. Immunological mechanism responsible for HBsAg nonresponsiveness

Literature describes a lot of phenomena which can explain the lack of a normal response to HBsAg after vaccination. Although some immunological mechanisms were analysed, an univocal explanation for the origin of this nonresponsiveness could not yet be found. Most publications on this topic can roughly be divided in two opposite opinions. A number of researchers (especially Japanese groups) propose the 'suppression hypothesis' wherein inheritance of nonresponsiveness would be dominant. However, most other groups argue against this hypothesis and suggest that the failure to respond to HBsAg is an MHC-determined recessive trait whereas the ability to mount an adequate antibody response is dominant. Both hypotheses are based on the observed MHC class II association in HBsAg vaccine nonresponsiveness. The MHC is thought to operate at least in two ways to mediate the immune response, via direct binding of processed peptides to class II MHC molecules as part of antigen presentation to $CD4^+$ helper T cells [185-187] and in the selection of T cells in the thymus or peripheral lymphoid organs with specific T cell antigen receptor (TCR) molecules [188,189]. In mice and humans there was an excellent correspondence between the in vivo anti-HBs level and in vitro T cell proliferation to HBsAg with nonresponders showing no T cell proliferation [190,191]. This and other observations suggest that the defect in nonresponder animals resides at the level of the T cell-APC interaction. This has also been shown for many other T-dependent immune responses [192,193].

II.5.4.1. The 'presentation defect' and 'determinant selection' hypotheses
In many studies in mouse [47] and in man [194] the lack of response to foreign antigens has been shown to be mediated primarily by the lack of binding of peptides to APC. There can be a failure of antigen uptake, processing, or interaction of processed antigen with MHC class II molecules. These mechanisms would lead to

defective antigen presentation with resultant failure to activate potentially activalable CD4^{+} T helper cells. For this mechanism, unresponsiveness would have to be recessive, requiring the defect to be specified by both of a nonresponder's MHC haplotypes.

T cells appear to recognize a limited repertoire of sites on a protein antigen. The estimate of diversity came from studies in mice of the recognition specificities for model protein antigens: myoglobin, cytochrome C or lysozyme [195,196]. In each instance it was found that T cells respond to a small number of regions of the protein molecule in a haplotype-specific manner. Therefore it was postulated that the function of MHC molecules is to specifically bind some peptides, but not all that are created during processing. This was called the 'determinant selection' hypothesis for MHC molecules.

Defective MHC binding is the most common explanation for lack of immune response to antigens such as hen egg lysozyme, ovalbumine, cytochrome C, and protein staphylococcal nuclease [197,198]. Since HBsAg gives rise to a limited number of immunogenic peptides [169], individuals with certain MHC alleles could fail to respond to the entire antigen, as their class II antigens can fail to bind to a major immunogenic peptide. Thus in its simplest form this hypothesis suggests that the non-responder status is the result of the inability of antigens to bind to MHC molecules leading to a failure of T cell stimulation.

It was demonstrated that low or nonresponders at the antibody level also failed to mount in vitro T cell lymphoproliferative responses to HBsAg. We [199] and others [200] performed T cell-APC mixing experiments using responder T cells and nonresponder APC (NR-APC), and concluded that defective MHC binding or antigen processing is not the basis for nonresponse. However, in these experiments we only analysed the potential of NR-APC to recall a T cell response and not the capability to induce a primary response. The presentation or priming capacity of dendritic cells from NR remains to be analysed.

General mechanisms for nonresponse, including deficient enzyme systems or enzyme inhibitors, may also be operative. However, the normal response to other vaccine (tetanus toxoid) or viral (Varicella Zoster) antigens in HBsAg nonresponders makes this unlikely.

II.5.4.2. The 'hole in the T cell repertoire' or 'shared epitope' hypothesis.
HBsAg contains a number of immunogenic peptides and is expected to be a good immunogen. However the observed lack of response to this antigen can be due to the absence or dysfunction of HBsAg-specific T cells. This defect could be due to a physical (thymic selection) or functional deletion (anergic state) of these T cells.

Strong binding of a peptide to MHC molecules is not enough for immunodominance. For a peptide derived from the bacteriophage lambda cI repressor, it could be shown that it was a strong binder but was not recognized by T cells. This peptide displayed homology to a self peptide of the Ia molecules which explained the absence of a T cell response [48]. Schaeffer et al.[201] studied 14 overlapping peptides that span the entire staphylococcal nuclease protein. Each of these peptides was incubated with Ia molecules and its binding was

determined. 30% of the peptides could bind Ia and, of these, 70% could elicit a T cell response. These findings provided evidence for 'holes in the T cell repertoire' and further showed that Ia molecules have a specific, though broadly permissive, capacity to bind peptides from a protein.

During thymic development many potential T cell receptor combinations are deleted and as a consequence there are 'holes in the T cell antigen recognition repertoire'. These holes are induced by specific MHC molecules during thymic education of T cells [201] or in the periphery. This mechanism would lead to unresponsiveness resident in T cells and not APC. One postulated mechanism in vivo by which the T cell defect in response to any antigen including HBsAg could have arisen is through molecular mimicry of a foreign epitope by protein encoded by self polymorphic non-MHC loci [186]. Evidence for such a mechanism was obtained in mouse experiments with a synthetic terpolymer [202]. Furthermore, numerous experiments have now been shown that MHC molecules can present self proteins [203]. For example, De Koster et al.[204] showed that one allele of a DP molecule could present an epitope of a DR molecule. This implies that self-proteins may be competing for foreigns for presentation.

A nice example of the involvement of molecular mimicry was described for a bovine leukemia virus (BLV)-associated pathology. The relationship between polymorphism of the bovine lymphocyte Ag (BoLA)-DRB3 gene and resistance and susceptibility to persistent lymphocytosis (PL) caused by BLV was investigated. A four amino acid homology between susceptibility-associated DRB3 alleles and BLV *pol* was found indicating that susceptibility to the subclinical progression of BLV infection could involve molecular mimicry [205].

II.5.4.3. The 'suppression' and 'Th1-Th2' hypothesis.

In this hypothesis the failure to respond to foreign antigens is mediated by the MHC-determined presence or excess of antigen-specific suppressor (CD8$^+$ or Th2 CD4$^+$) or CD8$^+$ CTL reacting against APC carrying a particular peptide [206] or against Th1 T helper cells of a particular specificity. Only one MHC haplotype would suffice for the effect, and inheritance of the nonresponse would be dominant.

A number of early studies compared the lymphocytic subsets and the PBL-proliferation to aspecific stimuli (mitogens) of responders and nonresponders to the HBsAg vaccine. Nowicki et al.[207] demonstrated that nonresponder (n=17) PBL posses significant higher amounts of CD2$^+$ (T11$^+$), CD57$^+$(HNK-1$^+$) and CD8$^+$ (T8$^+$) lymphocytes than responders (n=20). Furthermore, the nonresponder PBL show a reduced IgM and IgG production in vitro after PWM-stimulation. This led to the hypothesis that nonresponders have a larger popula-tion of T suppressor cells which influenced their normal immune response to the HB vaccine. This theory was confirmed by Japanse investigators [208], who postulated that, based on coculture experiments, nonresponse in the early faze is caused by a B cell defect and in a later fase is due to the presence of HBsAg-specific T sup-pressor cells. These authors found a dominantly expressed, MHC-linked CD8$^+$ T cell mediated suppression among the 20% of Japanese subjects who failed to respond to HBsAg immunisation. These findings were in contrast with observations by Egea et al.[190] who were unable to demonstrate any difference between R and

NR lymphocytic subsets, nor in proliferation upon PWM, PHA or TT. This study also analyzed the HBsAg-specific proliferation of T cells in the presence or absence of autologous CD8$^+$ T cells. No immune suppression could be demonstrated. Furthermore, removal of CD8$^+$ (suppressor) T cells did not result in T cell proliferation in nonresponder peripheral blood mononuclear cells [209]. These more recent results strongly suggest that neither cytotoxicity nor suppression via CD8$^+$ or Th2 cells plays an important role in the nonresponse to HBsAg. The Boston group postulated that nonresponders have a very specific (HBsAg-related) defect at the level of their antigen presentation and/or T cell stimulation.

II.5.4.4. The 'silent infection' hypothesis.

Recently a novel cause for nonresponsiveness to hepatitis B vaccines has been proposed. A Japanese [210] and a Chinese [211] study revealed that sera of some individuals who did not develop anti-HBs after repeated vaccination, were positive for HBV-DNA. Therefore it was proposed that immune tolerance or immune suppression, due to a latent low-level infection with the hepatitis B virus, may account for part of the nonresponsiveness to HB vaccination observed in Japan. We and others [199,209] could not detect HBV-DNA in sera from Caucasian nonresponders. The HBV-DNA detection method used was one of the most sensitive methods available today (Enzymun test/DNA detection that is applied on PCR-amplified specimens). We dare to conclude that the hypothesis formulated by Takahashi and Luo is only applicable for the Oriental population and may explain but a part of the observed nonresponsiveness.

II.5.5. Strategies to overcome nonresponsiveness to the HB vaccine

A first approach to overcome nonresponsiveness, is the design of a more immunogenic HBV envelope vaccine. This was done by adding preS1 and preS2 sequences to the conventional HBsAg vaccine. The underlying rationale for this approach can be found in mouse experiments. Studies in the murine model have shown that the immune response to the preS1 and preS2 regions of the HBsAg are regulated independently from the S region response, such that immunisation of S region nonresponder/preS2 region responder mice (H-2^s) with a preS2 containing particle (HBsAg/p33) circumvented nonresponse to the S region [212]. Similarly, immunisation of S and preS2 region nonresponder mice (H-2^f) with preS1 containing particles (HBsAg/p39) circumvented this nonresponsiveness and resulted in the production of antibodies against the S, preS2 and preS1 regions [109]. The independence of MHC-linked gene regulation of the immune response to the preS1, preS2 and S regions of HBsAg would assume fewer genetic nonresponders to a vaccine containing all 3 regions. Furthermore, in vitro neutralisation experiments and in vivo immunisation with preS synthetic peptides have shown that preS2 and preS1 specific antibodies in the absence of S region antibody can protect chimpanzees against infection [213]. The results of a study suggesting that preS1 antigen may be a strong T cell immunogen in humans warranted further studies to determine the necessity and/or advantages of the inclusion of preS sequences in third generation hepatitis B vaccines. Several experimental preS-containing vaccines have been produced and have

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been under evaluation for a number of years. Clinical vaccine trials yielded mixed results. The early human trials using preS2-containing vaccines proved safe and immunogenic, but indicated no distinct advantage over the existing S region-only vaccines [214,215]. Our own experience using a recombinant hepatitis B vaccine containing selected regions of the preS1 and preS2 sequences was rather deceiving [216,217]. However, in the same time frame, groups using preS2-containing [218,219] or preS1+preS2-containing [220] vaccines reported that the inclusion of the preS sequences conferred clear advantages. More recent trials using either preS2- or (preS1+preS2)-containing recombinant HBsAg vaccines seem to confirm that the preS sequences do confer distinct advantages including an early seroconversion to anti-preS2, which circumvents the problem of delayed appearance of anti-HBs antibodies in some vaccinees especially neonates, and efficacy in nonresponders to previous S region-only vaccines [221-224]. One reason for the more recent successes may be the inclusion of the preS1 region or components of preS1 sequences which were not included in the two earlier trials. Furthermore, different investigators have independently explored an alternative expression system for HBV surface proteins using the mammalian-derived Chinese hamster ovary (CHO) cell line [222-226]. To date, CHO-derived vaccines containing glycosylated and non-glycosylated S and preS2 proteins have been tested in mice and humans. Data suggest a markedly enhanced anti-HBs response and a lower rate of non- and hypo-responders. However, until now, the efficacy of preS-containing HBsAg vaccines remains a controversial subject. In different studies appropriate control groups were omitted. These 'third-generation' experimental vaccines will be judged mainly based on their potential property of inducing higher seroconversion rates, but not necessarily the higher anti-HBs titers. Finally, development of new improved vaccines should by no means affect the continually increasing distribution of the conventional vaccines for prevention of HBV worldwide.

A number of characteristics of the immune response to HBcAg suggest that this antigen may be useful for HBV and non-HBV vaccine development. For example: HBcAg has been shown to represent an efficient immunogen at the T-and B cell levels both in mice and man; the particulate HBcAg can elicit T cell independent as well as T cell dependent antibody responses; HBcAg is efficiently processed and presented by APCs; native HBcAg particles ensure strong T cell help not only to intrinsic B epitopes, but also to the non-HBcAg epitopes associated with it. Furthermore it was shown that HBcAg-specific Th cells can stimulate antibody production to HBcAg as well as to HBsAg, when both antigens are present within the HBV particle [227]. These characteristics suggested that HBcAg may be an ideal carrier moiety for B cell epitopes requiring additional Th function. Therefore, a number of HBV and non-HBV B cell epitopes have been chemically linked or fused by recombinant methods to HBcAg particles as a method to increase immunogenicity with significant success [228,229]. Studies of positional effects have demonstrated that an internal insertion into a dominant HBcAg-specific B cell site represents a superior location for enhanced antibody production [230]. Positive results were obtained using HBcAg as a carrier moiety for FMDV (foot-and-mouth disease virus) peptide, poliovirus type 1 C3 epitope, human rhinovirus type 2 epitope, FeLV (feline leukaemia virus) epitope, a HIV-gag sequence (human immunodeficiency virus),...[231]. The HBcAg was recently used as a carrier moiety for

malarial circumsporozoite antibody epitopes inserted into the surface loop structures. Efficient anti-malarial antibody and Th cell responses were elicited and the mice were protected against a challenge infection [232]. Instead of particulate HBcAg, it was shown that synthetic T cell sites derived from the HBcAg sequence can also function as carriers for B cell epitopes of the envelope of HBV and HIV ('peptide vaccine')[233]. These results indicate that HBcAg or synthetic T cell sites of HBcAg, or both, may represent an efficient T cell carrier moiety for multivalent vaccine development.

There has also been interest in the potential of DNA immunisation for prophylactic vaccination against HBV. In murine studies, intramuscular injection of plasmid DNA encoding the S region or the S region and the preS(2) region of the HBV envelope has been reported to induce anti-HBs and anti-preS(2) antibody production [234], potent $CD8^+$ CTL activity in a responder strain [235] and in a strain nonresponsive to other immunisation methods [236], and predominantly Th1-like $CD4^+$ responses [237]. Immunisation with plasmid DNA encoding HBsAg also elicited significant levels of anti-HBs in chimpanzees [238]. The humoral and the cellular responses occurred soon after DNA injection in mice (i.e. antibody, 1-2 weeks; CTL, 3-6 days) and persisted for 4-6 months after a single DNA injection. It has also been recently reported that the use of plasmids coexpressing IL2 and envelope proteins of HBV resulted in enhanced humoral and cellular immune responses [239]. Whether DNA-based immunisation proves to be more effective, less expensive or safer than recombinant proteins remains to be determined. However, DNA immunization may prove to be useful therapeutically in CH-B carriers.

In mice with genetically determined nonresponsiveness to HBsAg, several strategies which permit circumvention of this nonresponsiveness have been identified. Mice bearing the H-2^s haplotype are nonresponsive to intraperitoneal immunisation with 4 to 20 μg of HBsAg in Complete Freund's Adjuvant (CFA). Increasing the dose of antigen to 40 μg or changing the route of vaccine administration to footpad injection induced an anti-HBs response [240]. Immunisation of nonresponder mice with HBsAg conjugated to sheep erythrocytes (SRBC) resulted in an IgG anti-d specific response after a single dose but failed to induce an anti-a response after primary or secondary immunisation. Native HBsAg nonresponder SJL (H-2^s) mice produced low but detectable levels of anti-HBs when immunised with synthetic peptide analogues of HBsAg determinants [240]. These studies indicated that the B cell repertoire of nonresponder mice was intact and that nonresponsiveness could be circumvented by enhancing nonspecific T helper function.

The effect of additional vaccine doses on nonresponsiveness has been examined by several investigators. Wismans et al.[241] and Jilg et al.[242] found that intramuscular administration of 3 supplementary doses to healthy hyporesponders and nonresponders after a standard vaccination course induced a protective (≥10 IU/L) anti-HBs response in 75% of the subjects. In our study [217], using an identical experimental set-up, we found a seroprotection rate of 90% after 3 supplementary vaccine doses. Two Japanese groups [243,244] showed that intradermal injection of 5 μg HBsAg at 2-weekly intervals induced a positive (≥10 IU/L) anti-HBs response and a delayed-type hypersensitivity reaction in all subjects. These observations suggest that in humans (as well as in

mice) B cells with anti-HBs specificity are present in genetic nonresponders. These B cells can be incited to produce and secrete anti-HBs as soon as they receive adequate T cell help.

The use of immunomodulators or strong adjuvants is one of the many strategies to enhance the immune response. Adjuvants are thought to improve immune responses 1) by causing depot formation at the injection site (delay of immunogen release), 2) by acting as delivery vehicles for antigens, and 3) by acting as immunosti-mulators (improved presentation to T cells) [245,246]. Lipopolysaccharide (LPS) and more specifically its lipid A component has for long been known for its strong adjuvant effect. However toxicity precluded its use in a vaccine formulation. Ribi et al.[247] have shown however that a monophosphorylated form of lipid A (MPL) retains its adjuvant function and almost completely loses its endotoxin effects. More recently a 3-deacylated form of MPL has been shown to have a further decrease in its toxicity as tested in small animals, while retaining its immunopotentiating effect [248]. The commercially available 3-D-MPL is derived (3-deacylated) from the LPS of the gram negative Salmonella minnesota. Recent immunogenicity studies in mice have shown that MPL specifically induces antigen-specific Th1 type T cells and thereby significantly improves IFNγ production. Although animal experiments have shown that MPL mainly stimulates the cellular arm of the immunity, it also induces a stronger and faster anti-HBs antibody response. Vaccination trials with this new adjuvant are presently underway.

The effect of nonspecific immunostimulation of the murine immune response to HBsAg or related peptides has been examined in different studies. Coupling of the immunostimulatory sequences of interleukin 1ß (IL-1ß) in tandem with amino acids 12-32 from the preS1 sequence elicited an increased primary and secondary anti-S/12-32 response [249]. The humoral and cellular immune response of mice and guinea-pigs to HBsAg when alum-precipitated or administered with Syntex Adjuvant Formulation (SAF) were compared. SAF induced an increased and more consistent anti-HBs response in young mice and low responder strains. SAF allowed the reduction of the HBsAg dose to one-tenth of that required to elicit similar antibody responses by alum-adjuvanted HBsAg [250]. Algammulin, a new vaccine adjuvant comprising a stable suspension of 1 to 2 μm ovoids of the immune stimulant gamma inulin in which alum is embedded as a protein carrier, was shown to have a greater adjuvanticity than alum for HBsAg in mice [251].

Several strategies to overcome nonresponsiveness to HBsAg have been explored in haemodialysis patients. Increasing the antigen dose or administration of immune adjuvants such as interleukin-2 [252,253], interferon γ [254], interferon α [255] or thymopentin [256] improved the anti-HBs response. A recent report shows that treatment of haemodialysis patients with recombinant human erythropoietin increases antibody titers after hepatitis B vaccination [257]. This effect may be due to the avoidance of transfusion and a direct upregulation by erythropoietin of T and B cell function. Systematic studies exploring the effect of immunomodulating agents on the anti-HBs response in genetic nonresponders to HBsAg have not yet been performed.

II.5.6. HBV vaccination in newborns and infants

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The immunogenicity and safety of hepatitis B vaccines have been analysed in neonates and infants [258]. Inadequate responses to hepatitis B vaccine occur in approximately 5-15% of neonates [259]. In 1997 the Belgian governement has decided to include the hepatitis B vaccine into the standard infant immunisation programs, a decision that is however not yet implemented [260]. The persistence of protective anti-HBs levels in groups of children who had been immunised against hepatitis B 5 and 10 years earlier, during their first year of life, has been studied. In Italy, the absence of core antibody (anti-HBc) in responders to the vaccination shows the protective efficacy of both the recombinant yeast and plasma-derived vaccines. On the other hand, the presence of anti-HBc in some anti-HBs negative nonresponder subjects shows the susceptibility of these subjects to HBV infection [261]. The results of an Iranian study [262] indicate that a significant proportion of the nonresponder neonates to HBsAg can be induced to develop a protective and long-lasting antibody response by administration of a single additional vaccine dose. The disappearance, in some children, of protective levels of anti-HBs in the years following vaccination does not mean that all protection against HBV infection is lost. In fact, the trial showed that these children reacted immediately to booster stimulation, demonstrating a solid immunologic memory. Therefore, there may be no need to give booster injections when the vaccination of infants is carried out correctly [263].

Maternal-infant transmission and transmission in early childhood are important means by which the virus persists in the population. Each year, thousands of infants are born to women who are positive for HBsAg. These infants are at high risk for perinatal HBV infection and for chronic liver disease as adults. Without immunoprophylaxis, 70 to 90% of infants infected during the neonatal period or in the early infancy, will become chronic carriers (Table 3)[264]. In Africa, the majority of infections are acquired between the age of 6 months and 6 years. To identify newborns who require immunoprophylaxis to prevent perinatal HBV infection, all vaccine advisory groups have recommended routine HBsAg screening of all pregnant women during an early prenatal visit in each pregnancy [265]. In order to prevent vertical transmission of HBV, infants born to HBsAg-positive women, receive hepatitis B immune globulin (HBIG) and a first dose of hepatitis B vaccine at birth (passive-active hepatitis B immunisation), with follow-up doses of vaccine at ages 1 and 6 months. Vaccination alone is efficacious in preventing the development of HB in these children, but its efficacy increased with the concomitant use of HBIG [266-268]. It was shown that the protective efficacy of passive-active hepatitis B vaccination is mainly influenced by maternal HBV-DNA levels, and independent of time of starting active vaccination at birth or at 3 months of age. Additional measures are needed to protect neonates of highly viremic women [269,270]. Around 20% of newborns to mothers chronically infected by the HBV did not produce antibodies after correct vaccination. An in utero induced immune tolerance to low doses of HBsAg appears the most plausible hypothesis to explain this unresponsiveness to HBV vaccine [271].

II.5.7. Therapeutic vaccination

Vaccines can also be used to treat disease, not just to prevent it [147]. Since patients with a chronic hepatitis B

virus infection are characterized serologically by the absence of anti-HBs in the routinely used anti-HBs assays [272], it was appealing to consider HB vaccination for the treatment of these patients.

The rationale for the development of therapeutic vaccines in chronic hepatitis B is based on the hypothesis that vaccination should revert either a state of unresponsiveness due to tolerance or a state of disturbed immunity. Either of these states would be responsible for the chronic hepatitis. First, since the humoral response to HBsAg is enhanced by a vaccine coupled to a strong adjuvant, this may break the host's tolerance towards the virus and induce the production of anti-HBs antibodies. Second, CTL activity, through its recognition of viral antigen in the context of HLA-proteins at the liver membrane surface, is probably the main mechanism involved in the clearance of the virus via the destruction of infected hepatocytes.

In vitro and in vivo animal experiments have shown that some adjuvants are strong stimulants of cellular immunity, especially of the Th1 type [245]. These adjuvants will thus also enhance the production of IFNγ which has been shown to play an important role in the clearance of virus, by downregulating viral replication and facilitating destruction of infected hepatocytes. Inclusion of an immunostimulant should favor the induction of a Th1 T cell response against the HBV antigen. In addition, non HBV derived T cell epitopes should favor reversal of a state of anti-HBsAg T cell tolerance. Therefore, new vaccines could favor the establishment of an effective virus clearing immune response that would translate in seroconversion to anti-HBs, decrease of circulating HBV-DNA and, most probably, a transient rise in liver enzymes, the so-called flare-up, that heralds viral clearance.

The potential use of vaccines for the treatment of chronic hepatitis B patients has first been examined in HBsAg-transgenic mice. These mice were vaccinated and produced anti-HBs [273,274]. Later on the woodchuck- and Pecking Duck-model were tested [275,276].

Until now, at least three groups conducted human trials wherein chronically HBV-infected patients were vaccinated. The vaccines used were plasma-derived HBsAg [277,278] or recombinant HBsAg, containing preS2 sequences [279]. In the study by Dienstag et al.[277] there was no evidence that HBV vaccine was effective in eliminating HBV. By contrast, Pol et al.[148,279] reported that vaccination of chronic carriers with HBsAg containing the preS2 region lowered HBV replication and reduced HBV-DNA to undetectable levels. Wen et al.[278] immunised patients with chronic hepatitis B infection, with hepatitis B vaccine complexed to human hepatitis B immunoglobulin. They found that 9/14 patients became negative for serum HBV-DNA after three immunisations with this complex. All groups reported that vaccination appeared to be safe, with no side-effects, and no anti-HBs antibodies were detected after immunisation.

A synthetic peptide vaccine containing a CTL epitope of HBcAg (AA 18-27) and a Th epitope of tetanus toxoid (AA 830-843) is currently being investigated as a means of treating chronic hepatitis B infection [280]. Until now, results indicate that HBV-specific immune responses can be effectively elicited by synthetic immunogens at the level of T and B cell recognition, and that development of a synthetic HBV vaccine is feasable. Although a synthetic peptide-based HBV vaccine is probably not necessary in view of the effective

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recombinant vaccines, 'targeted' therapeutic uses for synthetic Th cell, CTL, and antibody recognition sites may be envisioned. In this context, the enhanced immunogenicity of HBcAg as compared to HBsAg, the ability of HBcAg to act as a T-cell-dependent antigen, and the relevance of HBcAg to protective immunity, suggest that HBcAg may represent an ideal carrier moiety for HBV antigens [231].

Whether therapeutic vaccination, by stimulating the cellular and humoral immunity and by presenting different epitopes to the immune system, could break the host's immune tolerance to the virus in chronic hepatitis B and induce a seroconversion, remains to be further explored. Although the development of vaccines with greater immunogenicity is very important in the future, care must be taken since the administration of exogenous HBsAg can induce CTL which increases liver cell destruction. Gene therapy [281,282] and immunotherapy (e.g. passive transfer of immune cells, bone marrow transplantation from hepatitis B immunized donors [283], cytokine therapy,...) are other possible therapeutic tools that can be useful in the future [284,285].

II.5.8. HBV vaccination and antigenic variability

II.5.8.1. Antigenic variability: general

Nucleotide substitutions in viral genomes can have several effects, including evasion of vaccine-induced or natural immunity, drug resistance, changes in pathogenicity, alterations in tissue or species tropism, and viral persistence. Viral genomes, particularly those of RNA viruses, are subject to inherent variability due to the incorrect incorporation of nucleotides during viral replication and an inefficient mutation repair system. The mutation rate is about 1 mutation in $1x10^3$ to $1x10^4$ nucleotides for each replication cycle of RNA viruses. DNA viruses have a much lower mutation rate of about 1 mutation in $1x10^5$ nucleotides per replication cycle [286,287]. The high mutation rate of RNA viruses is partly due to the properties of RNA polymerases/reverse transcriptases. In contrast to DNA polymerases, the RNA enzymes are not capable of correcting errors in nucleotide incorporation during polynucleotide synthesis ('lack of proof-reading function'). Thus, in the course of every viral infection - albeit with variable frequency in RNA and DNA viruses - mutations will occur, resulting in the emergence of variants. Obviously, to be competent, a variant must be able to infect efficiently, to replicate and to resist the defence mechanisms of the host.

The existence of HBV variants has been suspected for many years. This assumption was based on the detection of viral DNA and RNA in patients with chronic hepatitis in the absence of serological evidence of an HBV infection. In recent years, the systematic identification of HBV variants was made possible by the development of PCR, which enables the amplification of viral DNA or RNA in vitro. Although mutations in all viral genes and some regulatory genetic elements have been detected, two types of mutations seem to be very important and are referred to as precore mutants, and mutations in the common 'a' determinant of HBsAg [288-291].

The variability of HBV viruses is estimated to be intermediate between DNA- and RNA viruses [288]. HBV,

a DNA virus, replicates via an intermediate RNA stage with the use of reverse transcriptase. This enzyme is found in association with the virion and has no proofreading activity. It might therefore be expected that HBV viruses have a high mutation rate. But there are constraints on the ability of HBV to accept mutations without becoming non-viable. The genome is only about 3200 bases long, the smallest of any DNA virus that infects man, and all the genetic material codes for proteins with the regulatory elements being found within these coding regions. A mutation will thus most probably lead to a non-viable virus. This explains the rather small mutation-ratio that is seen in HBV viruses. Nevertheless, mutations in each of the four open reading frames were found in HBV viruses.

As documented in hepatitis B and C, genomes of hepatitis viruses frequently mutate during the course of disease. When such mutations occur in the region coding for antigenic epitopes recognized by the host's immune system, the viral change may lead to modulation of disease activity. For example, a single base substitution in the precore region of the HBV genome might induce activation of hepatitis from an immune tolerant state. Since HBV infections may persist for many years or even decades, mutations will accumulate over time and may become clinically relevant. The selection and accumulation of certain mutants depend not only on the time factor and on the replication activity, but also on the rate of elimination of wild-type viruses and the resistance towards antiviral mechanisms or drugs, amongst many other factors.

Mutations in the precore, core, surface, and polymerase genes have been studied in association with specific clinical conditions. The major protective epitope of HBV (the 'a' determinant) is believed to form two loops on the outside of the virus [292]. It is found in all known subtypes of HBV, and binds most of the anti-HBs found in hyperimmune serum. Mutations within this 'a' determinant have been described and most cases had a mutation from glycine to arginine at AA position 145 of HBsAg (named G145R)[289-293]. The mutant was identified for the first time in 1990 during the course of a passive (hepatitis B immune globulin)-active (HB vaccine) HBV immunisation program in southern Italy. Several neonates of HBV carrier mothers were observed to have become infected with HBV despite a primary response (adequate anti-HBs titer) to the HBsAg vaccine. Molecular analyses showed that one of these children was infected with a HBV mutant [289], exhibiting a defect in the S region of the HBV genome. This single amino acid change at position 145 probably induces a configurational change in the second external loop with loss of the group-specific antigenic 'a' determinant, the main target antigen of the vaccine response. By consequence the vaccine-induced antibodies cannot recognize the modified surface antigen anymore (Figure 14) [294]. This viral mutant was thus able to escape the immune surveillance, to replicate as a competent virus and thereby cause an infection despite the presence of anti-HBs antibodies ('vaccine escape mutant'). Since then, similar mutants have been detected in Japan, Spain, the United States, Great Britain, Singapore, Gambia. Presumably this mutation occurs worldwide [291]. Furthermore, this mutation has also been reported in patients after liver transplantation for HBV-related chronic liver disease who had received monoclonal anti-HBs antibodies in an attempt to prevent reinfection of the graft [295]. It is supposed that these mutants have appeared under pressure generated by anti-HBs antibodies, both

vaccine-induced and passively administered. Because HBV infection is diagnosed by the serological detection of antigens or antibodies, it is conceivable that, in addition to 'vaccine escape mutants', 'diagnosis escape mutants' will also exist. A change in the HBV antigenic structure can influence the sensitivity of these serological tests. Mutant HBsAg can be detected by some, but not all commercial kits [292]. The non-detection of HBsAg can lead to the transfusion of infectious blood or the transplantation of infected organs. Changes elsewhere in the 'a' determinant, or in other domains of the S protein (surface gene mutants) have been reported, although the significance of these findings is obscure [296].

Mutations have further been detected in the preS1 or preS2 regions of the HBV genome, but their clinical significance remains unknown [296]. Deletions rather than single-point mutations appear most often. Deletion mutants of up to one-half of the entire preS1 region were detected in 2 out of 10 individuals with chronic HBV infections following the initiation of interferon treatment. However, a mixture of wild-type and deleted forms was always observed [297]. In the study of Yamamoto et al. [298], deletions of up to 60 base pairs were found in at least 3 of 6 asymptomatic carriers, principally at the N-terminus of preS2. Furthermore, truncated integrated preS2-S gene sequences have a transcriptional transactivator function and appear to be associated with the development of HCC [299]. Whether these genomic changes result in a replication-competent virus is uncertain. The role of the immune response to the preS proteins has received less attention than the role of anti-HBS in viral neutralisation and elimination. However, preS-antibodies precede the development of anti-HBs during clearance of the virus. Furthermore, immunisation with just the amino terminal region of preS2 protects chimpanzees from HBV challenge [107]. So it is possible that mutations within the preS genes, as they have been described in patients with chronic HBV infection, can influence the normal viral neutralisation or elimination.

II.5.8.2. Implications for HBV vaccination

Since these mutant viruses can infect individuals with anti-HBs, it is very unlikely that current vaccines would protect against infection with this mutant. Until now, there are no data on the prevalence of these S gene mutants in the vaccinated population or the population at large. Their stability over time, their transmissibility within a population, the factors which promote their development, and thus the importance of these 'escape' mutants has yet to be established. Still, there is no evidence at this time that they pose a threat to immunization programs [38]. However, immune pressure on the population is occurring now as more countries take up universal vaccination.

It is very clear that G145R is the most stable and common variant seen after vaccination. The simplest solution is to vaccinate everybody with both G145- and R145- containing vaccines. The currently available vaccines are composed of S gene products derived from the wild type sequences. If S gene mutants are prevalent, future vaccines may need to be based upon both the S and preS gene product. In fact the preS region is a potent stimulant of T cell activity and may thus be a mechanism of enhancing immunoresponsiveness in patients with HBV variants.

Recently it was found that certain mutations in the polymerase gene of the HBV confer resistance to lamivudine [300]. Since the polymerase gene entirely overlaps the surface gene of HBV these mutations will also induce changes in the HBsAg gene. Thus the treatment of chronically infected HBV patients with lamivudine can seriously disturb the HBsAg-immunology post-treatment, and perhaps preclude a possible therapeutic vaccination.

II.6. References

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III. Outline and aims of this thesis: introduction to the experimental work

HBV causes acute and chronic liver diseases of variable severity by mechanisms that are thought to be largely immune mediated. Prevention of HBV-infection through vaccination has been routinely practiced and antibodies to the HBsAg have been shown to be effective in preventing infection after exposure to this agent. Following vaccination or natural acute HBV infection with recovery, significant titers of anti-HBs develop. However, approximately 5 to 10% of healthy vaccine recipients fail to produce protective levels of antibodies to the hepatitis B vaccine after standard immunisation. This phenomenon has been observed in all vaccine evaluation studies, irrespective of the HBsAg vaccine used. Although the cause of this humoral unresponsiveness remains unclear, it was repeatedly observed that NR not only display no, or low, humoral immune responses in vivo, but also fail to mount a lymphoproliferative response upon stimulation with HBsAg in vitro. Furthermore, a firm correlation between vaccine nonresponsiveness and certain HLA haplotypes was observed. The group of vaccinees that are genetically predestinated to become HBsAg vaccine nonresponders are discussed in this thesis.

The distinction between acute and chronic HBV infection is based on the clearance or persistence of envelope particles (HBsAg) and HBV-virions. Chronic HBV infections (5 to 10% of infected individuals) are almost certainly caused by a failure to mount an appropriate immune response to HBV. Until now, most studies reveal a defective production of neutralizing anti-HBs antibodies in these patients. Since the appearance of anti-HBs predicts complete recovery in acutely infected individuals, protects against reinfections in convalescent patients and protects against primary infections in vaccinees, a defect in this humoral response may be a key factor in establishing the carrier state. The causes of the defective anti-HBs production in patients with chronic HBV infections are completely unknown. Previous studies, based on vaccination data, have demonstrated that there is a critical requirement for T cells in the humoral response to HBsAg. In HBV carriers however, with one exception, the majority of studies has failed to demonstrate peripheral T lymphocyte sensitization to HBsAg.

Inadequate humoral and cellular immune responsiveness to HBsAg has thus been observed in vaccine nonresponders as well as in chronically infected individuals. Whether the mechanisms responsible for this HBsAg unresponsiveness are identical in both groups remains to be determined. The research presented in this thesis aimed at unravelling this HBsAg unresponsiveness and analysed the human cellular reaction which ensues after HBsAg-vaccination in healthy individuals. The methods used to analyse the immune response in vaccine NR can be helpful in the future to elucidate the etiology of HBsAg unresponsiveness in chronically infected individuals and can - in the long run - contribute to a better understanding of the pathogenesis of this disease.

The results of our research efforts are presented in chapter IV as a compilation of 8 manuscripts. Three have been published, one is in press, three have recently been submitted for publication and one is still in preparation. These manuscripts are organized in 4 sections according to the questions they address. In **Section IV.1.** we present the results of our study of the genetic factors that influence the human immune response to hepatitis B vaccines. This single paper provides answers to *immediate study objectives 1 and 2*. **Section IV.2.** encompasses

two manuscripts that summarize our exploration of the mechanisms of nonresponse to hepatitis B vaccines. These reports provide answers to *immediate study objectives 3 and 4*. **Section IV.3.** encompasses two manuscripts. One deals with the phenotypic and functional qualities of a series of HBsAg-specific T cell lines. The second paper reports on our analysis of the T cell recognition of HBsAg by PBMC from good responder vaccinees. This section tries to answer *immediate study objectives 5, 6 and 7*. **Section IV.4.** represents our contribution to the design and evaluation of 'improved' HB vaccines. Two companion papers summarize the results of two clinical evaluations of a candidate third generation HB vaccine containing selected preS1 and preS2 in addition to the entire S sequence. A third manuscript describes our immunological analysis of a delipidated HBsAg and demonstrates how delipidation improves T cell immunogenicity but reduces B cell immunogenicity to HBsAg. This section provides answers to *immediate study goals 8 and 9*.

The aims of this thesis were the following:

A. Immediate objectives

1. Determination of HLA-class II molecules linked with good and/or poor response to the HBsAg vaccine.

2. Analysis of interactions between HLA-factors involved in poor responsiveness.

3. Analysis of HBsAg-presentation capacity of APC derived from HB vaccine nonresponders.

4. Analysis of B7-costimulation in good and poor responders to the HBsAg.

5. Identification of immuno-dominant T cell epitopes of the HBsAg at the population level.

6. Development of HBsAg-specific T cell lines in HBsAg good and poor responders.

7. Identification of epitope- and HLA-restriction of these HBsAg-specific T cell lines.

8. Analysis of the immunogenicity of a preS-containing HBsAg vaccine.

9. Analysis of the immunogenicity of delipidated HBsAg.

B. Long-term objectives

* Elucidation of the etiology of HBsAg-unresponsiveness in HB vaccine nonresponders.

* Elucidation of the etiology of HBsAg-unresponsiveness in HB chronic carriers.

* Development of a new, more immunogenic vaccine that induces a protective anti-HBs response in ALL vaccinated individuals, if possible with fewer vaccine doses.

* Development of a therapeutic vaccine to cure chronically infected patients.

IV. Publications

IV.1. Genetic factors influence the human immune response to hepatitis B vaccines

Response to hepatitis B vaccine: multiple HLA genes are involved

Isabelle N. Desombere, Annick Willems, and Geert G. Leroux-Roels

Running Title: HLA and response to hepatitis B vaccine

Key words: Hepatitis B surface antigen, Hepatitis B vaccines, HLA antigens, immune response, nonresponse

Remark: The publication is presented in its 'preprint' form. We apologize for the minor typographic errors.

IV.2. Analysis of the mechanisms of nonresponse to HBsAg in vaccinees

IV.2.1. Nonresponders to hepatitis B vaccine can present envelope particles to T lymphocytes

Isabelle Desombere, Pierre Hauser, Rudi Rossau, Joseph Paradijs, and Geert Leroux-Roels

Running title: Nonresponsiveness to hepatitis B vaccines

Key words: Hepatitis B surface antigen, Hepatitis B vaccines, HLA-D-Antigens, HLA-DR2-Antigen, HLA-DR7-antigen, immune response, nonresponse.

IV.2.2. Analysis of HBsAg-presentation and B7-costimulation in nonresponders to the hepatitis B vaccine

Isabelle Desombere, Yvonne Gijbels, Tinghua Cao, and Geert Leroux-Roels

Running Title: Nonresponsiveness to hepatitis B vaccines

Key words: Hepatitis B surface antigen, Hepatitis B vaccines, HLA-DR3-Antigen, B7.1., B7.2., immune response, nonresponse.

In preparation

Remark: This article is presented in its 'preparation' form. Tables and figures are added at the end of the text.

IV.3. T helper epitopes of the hepatitis B envelope proteins

IV.3.1. T cell epitopes of hepatitis B virus envelope (HBVenv) proteins recognized by good responders to HBVenv vaccines

Isabelle Desombere, Geert Leroux-Roels, Yvonne Gijbels, and Andrea Verwulgen.

Running title: HBVenv-specific T cell epitopes

Key words: T cell epitopes, HBsAg, synthetic peptides, HLA-restriction

Submitted for publication

Remark: This publication is presented in its 'submission' form. Tables are added at the end of the text.

IV.3.2. Characterization of different hepatitis B specific T cell lines

Isabelle Desombere, Yvonne Gijbels, Andrea Verwulgen, Els Van Hecke and Geert Leroux-Roels

Running title: HBsAg-specific T cell lines

Key words: T cell epitopes, HBsAg, synthetic peptides, HLA-restriction

Submitted for publication

Remark: This publication is presented in its submission form. Tables and figures are added at the end of the text.

IV.4. In search of more immunogenic HBsAg vaccines

IV.4.1. Hepatitis B vaccine containing surface antigen and selected preS1 and preS2 sequences. 1. Safety and immunogenicity in young, healthy adults.

Geert Leroux-Roels, Isabelle Desombere, Guido De Tollenaere, Marie-Anne Petit, Pierre Desmons, Pierre Hauser, Andrée Delem, Danny De Grave, and Assad Safary

Running Title: Evaluation of preS-containing HBV vaccine.

Key words: hepatitis B vaccine, preS region, humoral and cellular immune response

IV.4.2. Hepatitis B vaccine containing surface antigen and selected preS1 and preS2 sequences. 2. Immunogenicity in poor responders to hepatitis B vaccines.

Geert Leroux-Roels, Isabelle Desombere, Luc Cobbaut, Marie-Anne Petit, Pierre Desmons, Pierre Hauser, Andrée Delem, Danny De Grave, and Assad Safary

Running Title: Evaluation of preS-containing HBV vaccine

Key words: hepatitis B vaccine, poor-responsiveness, preS region, humoral and cellular immune response

IV.4.3. Partial delipidation improves the T cell immunogenicity of hepatitis B surface antigen

Isabelle Desombere, Geert Leroux-Roels, Martine Wettendorf, Annick Willems, Yvonne Gijbels, and Andrea Verwulgen.

Running title: Delipidation of HBsAg improves its T cell immunogenicity

Key words: Hepatitis B vaccines, HBsAg, cellular immune response, delipidation

Submitted for publication

Remark: This publication is presented in its 'submission' form. Tables and figures are added at the end of the text.

V. Conclusions and general discussion

V.1. Conclusions

The experimental data obtained in this study have led us to the following conclusions:

1. The human immune response to HBsAg is influenced by genes located in the MHC class II region.

Although the mechanisms causing inadequate immune responses to HBsAg-vaccines in 10% of healthy young adults remain largely unknown, variations in the immune response are often associated with polymorphism of the MHC. In mice, the anti-HBs production is controlled by the H-2 system. An increased incidence of poor responsiveness in subjects with HLA-*DR3* and/or *-DR7* haplotypes has been documented, suggesting that HLA-DR-linked genes may regulate the human response to HBsAg. In chapter IV.1 of this thesis we analyzed the association between MHC and the human humoral immune response to the hepatitis B vaccine. By correlating HLA haplotypes of vaccinees to their HBsAg response patterns, we were able to identify HLA markers associated with good response (HLA-DRB1*010*, *-DR5*, -DPB1*040*, -DQB1*0301, and -DQB1*0501) and HLA-markers associated with non/poor response to HBsAg (HLA-DRB1*07, -DPB1*1101, and -DQB1*020*). This was the first indication that HLA-DP molecules, besides HLA-DR and -DQ, influence the human immune response to HBsAg. We further found that HLA-DPB1*02 was negatively associated with responsiveness when it occurred in association with haplotype DRB1*0701/DRB4*0101-DQB1*020*, and DRB4*0101 was negatively associated with responsiveness when it occurred in association with haplotype DRB1*0301/DRB3*0101-DQB1*020*. Clearly, interactions between HLA molecules that are not in linkage disequilibrium contribute to poor responsiveness.

Homozygosity for HLA-*DR3*, *-DR7* and *-DQ2* tended to be positively associated with nonresponsiveness. Seven HLA-*DR7*, 7 HLA-*DR3* and 19 HLA-*DQ2* homozygous individuals were observed in the non/poor responder population, whereas only 1 homozygous *DR7*, 1 homozygous *DR3* and 2 homozygous *DQ2* individuals were observed among good responders (Figure 15). Recently, heterozygosity at these HLA class II loci (HLA-DR and -DQ) was shown to be associated with protection from persistent HBV infection [1].

These data represent strong evidence for a role of MHC class II molecules in HBsAg-vaccine unresponsiveness.

2. The nonresponsiveness to HBsAg vaccine in HLA-DR7^{+} and/or -DR3^{+} subjects does not reside in a defect of HBsAg presentation of their PBMC.

In chapter IV.2. of this thesis we present the studies we have performed in an attempt to unravel the mechanism(s) responsible for the immunological unresponsiveness to HBsAg. Theoretically, nonresponsiveness to HBsAg vaccines may be caused by a defect (alone or in combination) at each of the numerous stages involved in the human immune response. However, the correlation between in vivo humoral and in vitro cellular responses and the known critical requirement for T cells in the anti-HBs response suggests that the inadequate

antibody production in vivo may be caused by a lack of adequate T cell help. For these reasons we focused on the initial processes of the immunological cascade, namely the capacity of APC to present HBsAg. Since several reports described an increased incidence of nonresponsiveness in subjects with the HLA-*DR3* and/or -*DR7* haplotype, we concentrated our research on individuals expressing these HLA molecules. We were able to demonstrate that our inability to detect a T cell response in vaccine NR was not caused by a defective HBsAg-presentation of their APC. HBsAg-presentation capacity was analyzed in HLA-*DR2*^{+} ,-*DR7*^{+} and -*DR3*^{+} nonresponders to the hepatitis B vaccine. This functional analysis of HBsAg-processing and -presentation was performed with a series of HBsAg-specific T cell lines or clones with known antigen fine-specificity (peptide fragment) and HLA-restriction. Antigen was presented to these T cells through haploidentical responder and nonresponder APC. Since T cell clones/lines do not require costimulation for their proliferation, a defect at the B7-CD28 level could not be excluded in these individuals. For this reason, we further analyzed the quality of the costimulatory signals B7.1. and B7.2. in these individuals.

3. B7.2.-costimulation is probably not deficient in NR

In this thesis we demonstrate that CD86, which is primarily involved in the induction of an optimal T response with recall antigens and which is normally constitutively expressed on human peripheral blood monocytes, was not downregulated after culturing PBMC from NR with HBsAg. This was analyzed in an HLA-*DR3* and -*DR7* homozygous context. We conclude that B7-costimulation is probably not deficient in non/poor responders to the hepatitis B vaccine.

4. Several HBsAg-derived T helper epitopes are involved in responsiveness to hepatitis B vaccines.

In the past, our group has clearly demonstrated that there is a critical requirement for T cells in the humoral response to HBsAg. Since a close correlation between the in vivo humoral (anti-HBs production) and the in vitro cellular (T cell proliferation) responses to HBsAg has been demonstrated in HB vaccine recipients and in patients with an acute HBV infection, it was tempting to speculate that the magnitude of anti-HBs responses is largely determined by the quality and magnitude of the HBsAg-specific T cell responses. For these reasons we embarked on a more detailed analysis of the human T cell response to HBsAg in vaccine recipients. We thus identified a number of immunodominant T epitopes in the HBsAg. Six of them were recognized preferentially (immuno-dominant epitopes) by a group of 32 high-responder vaccinees (population-level). Other T cell epitopes of HBsAg were identified by analyzing the fine specificity of a series of HBsAg-specific T cell lines. The identification of immunodominant T cell epitopes can be helpful in understanding the etiology of HBsAg unresponsiveness and in the development of new vaccines.

In chapter IV.3. we describe our efforts to map T cell epitopes on the HBsAg. In Figure 16 an updated T cell epitope map of the HBsAg is given. It clearly shows that numerous T cell epitopes are scattered over the HBsAg sequence. This leads us to the concept that in the APC a large number of different peptides are

generated by processing of HBsAg. A substantial number of these peptides are capable to interact with the HLA class II molecules expressed by that cell. Despite the numerous T cell epitopes that were identified, HBsAg remains a notoriously poor T cell immunogen (e.g. compared to HBcAg), inducing only a weak T cell proliferative response with short-lived memory.

5. The first hydrophobic region of the HBsAg is highly immunogenic at the T-cell level in man.

The majority of HBsAg-reactive T cell clones/lines (10 out of 12) recognize sequences located in the first hydrophobic region. Figure 16 also shows that especially the first hydrophobic region HBsAg contains a cluster of several overlapping T cell epitopes. Whether this immunogenicity resides in the AA composition of this domain or in its topography in the HBsAg particle remains to be determined.

6. DRB1*0701 and DPB1*0402 interact with and present the same AA sequence in HBsAg

HLA-DP0402 and -DR0701 can both present the HBsAg-derived peptide 193-207. The described HLA binding motifs for these HLA molecules can be found in this AA sequence. These results indicate that sequence 193-207 contains a cluster of at least two overlapping T cell epitopes. Surprisingly, this peptide can be presented by an HLA molecule associated with a good humoral vaccine response (HLA-DP0402), and with an HLA molecule known to be associated with non/poor responsiveness. The impact of this observation, however, remains unknown.

7. Poor responders to HB vaccine recognize fewer T cell epitopes than good responders

A limited analysis of the T cell epitopes of HBsAg recognized by good and poor responders to HBsAg vaccine revealed that good responders generally recognize multiple sites within the HBsAg sequence. Numerous T cells with different specificities are thus triggered upon stimulation with HBsAg and this explains the vigourous nature of the response in good responders. It is extremely difficult to raise T cell lines or clones from poor responders and these turn out to be directed towards one or a very limited number of epitopes. The pauciclonality of the T cell response in poor responders probably explains the absence and weakness of their T cells, and overall response to the vaccine.

8. Addition of selected preS-sequences to existing HB vaccines did not enhance the in vivo humoral anti-HBs response in young healthy adults, nor in established poor responders.

A number of target populations do not readily seroconvert when immunized with the conventional HBV vaccine. These groups include immunosuppressed patients, chronic hemodialysis patients, and elderly people. Furthermore, 5 to 10% of healthy young adults are unresponsive to HBsAg. These different groups make it worthwhile to continue the search for more immunogenic HB vaccines. Furthermore the reduction of the number of vaccine doses can decrease the costs of vaccination and increase the compliance. In an attempt to

circumvent HBsAg-vaccine unresponsiveness, we analysed whether modifications of the hepatitis B vaccine can improve the induced immune response. Based on clinical observations in natural HBV infection and experimental evidence obtained in mice and chimpansees, SmithKline Beecham Biologicals has designed a recombinant HB vaccine containing selected preS1- and preS2 sequences in addition to the full length HBsAg sequence. This molecule was called L* and was coexpressed with HBsAg in S. cerevisiae, called S-L*. In one study we compared the safety and immunogenicity of SL* with that of Engerix-B in two groups of young, healthy adults. In a second study we examined whether S-L* induced a faster or higher anti-HBs response as compared to Engerix-B within a group of 32 healthy adults with an established poor response to existing HB vaccines. These studies led to the following conclusions:

   * Addition of the selected preS sequences to S did not enhance the in vivo humoral anti-HBs response in young healthy adults, but improved the in vitro stimulating capacity of the antigen (L*) in S-L* primed subjects.

   * Addition of preS sequences to S did not enhance the in vivo humoral anti-HBs response in established poor responders.

   * The addition of three additional vaccine doses, irrespective of their preS content, induces seroprotective anti-HBs levels in 91% of the originally poor-responding vaccinees.

   * In vitro proliferative response to HBV envelope antigens were only observed in poor-responding subjects displaying anti-HBs titers > 1000 IU/l after the third additional vaccine dose.

9. Partial delipidation improves the T cell immunogenicity of hepatitis B surface antigen.

   The remarkable composition and variable immunogenicity of the HBsAg incited us to analyse whether the delipidation of HBsAg particles alters the immunogenicity of the antigen. Earlier publications reported that delipidation of HBsAg with detergents or phospholipase C, could alter the immunogenicity of the HBsAg particle. The underlying rationale was that the high lipid content (+/- 40%) of the HBsAg particles might represent an obstacle to the successful processing and presentation of the antigen on top of the constraints already imposed upon the system by HLA restriction. Partial delipidation of the particles could favour the immunogenicity of the particle by facilitating the lipid digestion and enhancing the accessibility for processing of the protein moiety. To examine this issue we treated HBsAg with the non-denaturing detergent ß-D-octyl glucoside (chapter IV.4.3.) and analyzed the immunogenicity of these altered particles in vitro as well as in vivo. These studies led to the following conclusions:

   * The delipidated particle induced better T cell responses than its untreated counterpart and this as well in good responders, nonresponder vaccinees and chronic hepatitis patients.

   * Delipidated HBsAg alone has a significantly reduced B cell immunogenicity (antibody recognition) as compared to native HBsAg.

   * When native and delipidated particles were mixed, the amount of native HBsAg needed to mount a normal response, can be considerably reduced.

V.2. General discussion

Whether a vaccine will induce an adequate immune response in a vaccinee will depend on qualities of the host and on the intrinsic properties of the vaccine. In this thesis we studied in detail the T cell response against a well defined structural protein of the HBV, the hepatitis B surface antigen (HBsAg), which is currently used as hepatitis B vaccine. Immune response to HBsAg was studied in both good and poor responders to this vaccine.

Several immunological mechanisms have been put forward to explain nonresponsiveness to the T-dependent HBsAg, but the exact nature of this problem remains unidentified. Recent data enfeeble the 'suppression' hypothesis for nonresponsiveness, in which HBsAg-recognition induces negative signals ('suppression') which selectively suppress a normal humoral anti-HBs response. Also the hypothesis suggesting a 'B cell repertoire defect', in which B cells recognizing HBsAg and/or synthesize the appropriate anti-HBs molecules (immunoglobulines) are absent, became very unlikely considering the fact that most NR to HBsAg (human and mouse) were able to produce anti-HBs when stimulated more vigorously (sometimes up to 12 doses are needed). Today, several observations suggest that the defect in nonresponders resides at the level of the T cell-APC interaction, as has been shown for many other T-dependent immune responses. In mice and humans there was an excellent correspondence between the in vivo anti-HBs level and in vitro T cell proliferation to HBsAg, with nonresponders showing no T cell proliferation; in other words, HBsAg-specific B and T cell responses are closely correlated. Since the generation of an anti-HBs response requires cooperation between HBsAg-specific T and B lymphocytes, the absence or reduction of T cells may lead to a defective or absent anti-HBs response. Furthermore, there are strong arguments for a role of HLA-class II molecules in the human immune response to HBsAg; genes associated with good and poor response as well as gene complementation has been observed. However, the precise linkage between HLA and HBsAg responsiveness is not understood.

The quality of particular HLA haplotypes (e.g. **HLA-*DR3*, *-DR7*, *-DQ2*,..**) can mediate the immune response via direct binding of processed peptides to HLA class II molecules as part of antigen presentation to CD4$^+$ helper T cells. Thus particular HLA molecules can be responsible for a deficient HBsAg-presentation, i.e. peptides generated during processing are unable to fit in the peptide binding groove of MHC class II molecules. By consequence, no peptide/MHC complexes will be presented at the surface of the APC. We speculate that this occurs for **HLA-*DQ2*** molecules. We assume that no HBsAg-derived peptides can stably interact with these molecules. This is based on the finding that the published *DQ2*-binding motifs, although the peptide-binding specificities of human HLA-*DQ* MHC class II proteins are still controversial, do not occur in the entire HBsAg-sequence [2]. Furthermore, we nor others were able to demonstrate the existence of *DQ2*-restricted HBsAg-specific T cells.

Surprisingly, **HLA-*DR3*** and **-*DR7*** molecules can adequately present HBsAg to T lymphocytes and no B7-costimulation defect could be observed in these NR. A HBsAg-specific deficiency in the T cell population

seems the most likely explanation for these NR. It remains to be determined whether these cells are absent, fail to recognize the antigen or are anergized. A hole in the T cell repertoire by elimination or anergization of HBsAg-specific T cells during thymic or post-thymic development and maturation could lead to the absence of T cell help for HBsAg-specific B cells in these individuals. It is known that HLA molecules can operate in a second way to mediate the immune response, i.e. through the selection of T cells in the thymus or peripheral lymphoid organs with specific T cell antigen receptor (TCR) molecules. The available T cell receptor repertoire is partly determined by the positive and negative selection in the thymus. In this selection, T cells reactive with self antigens are deleted. We reasoned that the majority of *DR3-* and *DR7*-restricted HBsAg-derived T cell epitopes are not recognized as foreign because they contain or mimic self-related sequence patterns.

In contrast to e.g. *DR1*, for which many binding motifs were found in the HBsAg-sequence, only few *DR3* and *DR7* binding motifs were identified. We thus assume that the diversity of *DR3*/peptide and *DR7*/peptide combinations is very limited. This makes the 'hole in the T cell repertoire'-hypothesis acceptable.

Remarkably, after numerous attemps in many different individuals, we were able to generate *DR3-* and *DR7*-restricted HBsAg-specific T cell lines. It is conceivable that, in some individuals, there are T cells recognizing the (*DR3*/peptide or *DR7*/peptide) complex. This can be due to the absence of the responsible self-protein in these individuals. In this context, the finding of gene complementation in HBsAg-nonresponsiveness can be valuable.

In conclusion, some HLA molecules associated with nonresponsiveness are unable to present HBsAg-peptides, others can, but in combination with certain HBsAg-mimicking molecules (polymorphic self, such as HLA molecules, or non-self, such as bacterial proteins), there is a hole in the T cell repertoire for recognition of these HLA molecules and no or very little T cells recognizing these complexes will be found in the periphery. If this hypothesis turns out to be correct, it remains to be established which is (are) the 'tolerogenic' self-antigen(s) that is (are) interacting with *DR3* and *DR7*. In an attempt to unravel this molecular mimicry, all currently known polymorphisms of the human MHC are at present screened for AA homology with HBsAg (or known T cell epitopes) using a protein database (Swissprot).

We assume that the magnitude of the humoral anti-HBs response of one individual is largely dictated by the strength of his/her HBsAg-specific T cell response. The latter will depend on the quantity and quality of the (HLA-class II/HBsAg-derived peptides) complexes presented on the surface of the APC's. When these complexes are made of HLA molecules associated with good response (HLA-*DR1*, HLA-*DP4*,..) a vigourous response follows. If the complexes are of poor responder nature (HLA-*DR3*,-*DR7*, *DP11*,..) no or a very poor response ensues. Mixed presentation of poor and good responder HLA molecules tends to be sufficient to induce adequate responses. This view is supported by our observation of polyclonal and polyspecific T cell responses in good responder vaccinees: numerous AA sequences are recognized in association with different HLA-restricting elements. This is also termed antigen and epitope spreading. The ability to present a wide range of peptides leads to a more effective immune response. It can be accomplished through the availability of

multiple 'participating' HLA molecules. On the other hand, HBsAg-specific T cell responses in poor responders are difficult to reveal and are restricted towards a limited number of epitopes. This pauciclonality can be explained by the scarceness of 'participating' HLA molecules. We assume that normally responders posses a broad T cell repertoire, and NR a narrow one. The limited heterogeneity of the responses observed in NR can partly be attributed to the frequently observed homozygozity in these individuals. Especially individuals homozygous for HLA-*DR3*, -*DR7* or -*DQ2* are known to be **non**responders.

It can be concluded that T cells play a very important role in the generation of an adequate response towards the HBsAg. A defect in the HBsAg-specific T cell population might therefore be the most likely explanation for the existence of nonresponsiveness. Although the mechanisms mentioned above, might be valid, some arguments remain speculative and need to be confirmed. Therefore, further studies are needed to explore the exact nature of this T cell deficiency in HBV vaccine recipients. Further studies could be focussed on the role of professional APC, i.e. dendritic cells, in the generation of NR, since these cells not only play a role in the induction of T and B cell responses but also in the induction of anergy and tolerance. The extraction of HBsAg-derived peptides out of HLA molecules and their identification can also shed a new light on the mechanism of HBsAg-nonresponsiveness.

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Abbreviations

AA	amino acid
Ab	antibody
Ag	antigen
ALT	alanine aminotransferase
anti-HBc	antibody to hepatitis B core antigen
anti-HBe	antibody to hepatitis B e antigen
anti-HBs	antibody to hepatitis B surface antigen
APC	antigen-presenting cell
AST	aspartine aminotransferase
BLV	bovine leukemia virus
BSA	bovine serum albumin
CAH	chronic active hepatitis
CC	chronic hepatitis B virus carrier
CD	cluster of differentiation
CFA	complete Freunds adjuvant
CH	chronic hepatitis
CPH	chronic persistent hepatitis
cpm	counts per minute
CsCl	cesium chloride
CTL	cytotoxic (cytolytic) T lymphocyte(s)
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
g	gravity
G145R	mutation from glycine to arginine at AA position 145 of HBsAg
gD2	Herpes simplex glycoprotein D2
GMT	geometric mean titer
gp	glycoprotein
H-2	major histocompatibility complex of mice
HB	hepatitis B
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBIG	hepatitis B immune globulin
HBsAg	hepatitis B surface antigen
HBSS	Hanks balanced salt solution
HBV	hepatitis B virus
HBVenv	hepatitis B virus envelope
HBxAg	hepatitis B x antigen
HCC	hepatocellulair carcinoma
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HR	high/good responder vaccine recipient
HSV	herpes simplex virus
^{3}H-TdR	tritiated thymidine deoxyribose
Ia	MHC class II histocompatibility molecule of mice
IFA	incomplete Freunds adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular
Ir	immune response
ISI	integrated stimulation index
IU	international unit
kD	kilodalton
LiPA	Line Probe Assay
LPS	lipopolysaccharide
LTT	Lymphocyte transformation test
2-ME	ß-mercaptoethanol
mAb	monoclonal antibody
MHC	major histocompatibility complex
MHR	major hydrophilic region
MLR	mixed lymphocyte reaction
MW	molecular weight
n	number in study or group
NA	not applicable
ND	not determined
NK	natural killer
NR	non/poor responder vaccine recipient
n.s.	not significant
OG	ß-D-octyl glucoside
ORF	open reading frame
P	probability
PBL	peripheral blood lymphocyte(s)
PBMC	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
Pc	corrected P value
PCR	polymerase chain reaction
PDV	plasma-derived hepatitis B vaccines
PE	phycoerythrin
PHA	phytohemagglutinin
PHC	primary hepatocellular carcinoma
PI	propidium iodide
PWM	pokeweed mitogen
r	recombinant
R	responder vaccine recipient
rad	radiation

RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RR	relative risk
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacryl-amide gel electrophoresis
SI	stimulation index
SSC	side scatter
TCR	T cell receptor
Th cells	T helper cells
TNF	tumor necrosis factor
TT	tetanus toxoid
U	unit
UV	ultraviolet
v/v	volume/volume
VZAg	varicella/zoster virus antigen
WHO	World Health Organisation
w/v	weight/volume
YDV	yeast-derived hepatitis B vaccines

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Figure 1: *World-wide endemicity of hepatitis B virus. Adapted from WHO Europe - Viral Hepatitis Prevention Board- European Occupational Health Series n°8 [10].*

Figure 2: *The genomic map of the hepatitis B virus. The viral DNA is partially double-stranded (red circle). The long strand of fixed length (blue circle) encodes seven proteins from four overlapping reading frames (surface [S], core [C], polymerase [P], and the X gene [X], shown as large arrows, and three upstream regions (preC, preS1, and preS2). A protein is covalently linked to the 5'end of the long strand (hatched blue oval) and a short oligoribonucleotide at the 5'end of the short strand (red zigzag). The size of each segment is shown in parentheses. The diagram is adapted from Lee WM, 1997, [4].*

Figure 3: *Structure of the hepatitis B virion.*

Figure 4: *Representation of the antigen and polypeptide composition of envelope and nucleocapsid proteins of intact virions and spherical and filamentous subviral particles. L, large; M, middle; S, S-region (adapted from Milich DR, 1997, [18])*

Figure 5: *The three different forms of hepatitis B surface antigen. Details of the surface protein structure are shown (adapted from Lee WM, 1997, [4]).*

Figure 6a: Secondary structure model for the arrangement of the small envelope protein in 22 nm particles. The predicted four membrane spanning segments project the N- and C-termini of S on the outer surface of the particle. The partial glycosylation at position 146 is indicated by (¥)(adapted from Stirk HJ, et al., 1992, [25]).

Figure 6b: Proposed model of major hydrophilic region (MHR) indicating cysteine (black circles) to cysteine disulphide bridging (∖∕∖). The five antigenic regions are labelled HBs1 through 5 (adapted from Wallace LA, and WF Carman, 1997,

[9]).

Figure 7: *Estimated outcome of hepatitis B infection in adults. Annual estimated cases in the WHO European region. Adapted from Van Damme P, et al., 1995, [37].*

Figure 8*: Schematic representation of the structural features of major histocompatibility complex (MHC) class I and class II molecules. MHC class I molecules are anchored in the membrane by a single transmembrane segment contained in the 45 kD α-chain. The MHC class I α-chain is noncovalently associated with ß2-microglobulin. There are four external domains, three of which contain intramolecular disulfide bonds, as shown. In contrast, MHC class II molecules consist of noncovalently associated α- and ß-chains, both of which are anchored within the membrane. The overall domain organisation, however, is highly similar. Glycosylation sites on both molecules are indicated by a solid circle.*

Figure 9*: Peptide (red) bound in the cleft of a HLA-class II molecule (blue) from human cells. This 'top view', probably as seen by T cells, is based on the X-ray crystallographic analysis of Brown and colleagues, 1993, [51]. The α1 domain helix*

is represented on top, the ß1 domain helix on the bottom. The ß-sheet is located on the bottom of the cleft.

Figure 10: Pathways of HLA class I (left panel) and II molecule (right panel) assembly and antigen processing. In the class I pathway, endogenously processed peptide is carried to the endoplasmic reticulum (ER) by putative peptide transporter molecules. There, the class I heavy chain and ß2-microglobulin combine to form the class I molecule. This dimer binds the processed peptide; binding of the peptide may occur as the class I molecule is being assembled. The class I-peptide complex then traffics through the Golgi and post-Golgi compartments and moves to the cell surface. At the surface, it is available to interact with T cells, facilitated by interaction with the CD8 molecule, which is to bind to the α3 domain of class I molecule. This compartmentalised pathway apparently restricts the binding of class I molecules primarily to endogenous peptides; the binding interaction between the class I heavy chain and the CD8 molecule directs class I endogenous peptide complexes to interact with CD8+ T cells. In the class II pathway, class II α- and ß-chains are assembled in the ER along with the invariant chain, which is thought to interact in such a way that peptide cannot bind to the antigen-binding groove. Thus most endogenously processed peptides, which are transported to the ER are unable to bind to class II molecules. After transport through the Golgi and post-Golgi compartments, the class II α-ß invariant chain complex moves to an acidic endosome, where the invariant chain is proteolytically cleaved and released from the class II molecule. At this point, exogenously derived peptides which have been brought to the endosome through fusion of phagolysosomes or recycling endosomes may now bind to the class II molecule. This class II molecule-peptide complex is then transported to the cell surface, where it is available to interact with an appropriate T cell. The structure of the class II

molecule allows preferential interaction with the CD4 molecule, which facilitates recognition between the class II peptide complex and an appropriate TCR.

Figure 11: *Schematic presentation of T lymphocyte recognition of MHC class II molecule and peptide. The principal co-stimulatory signal expressed on professional antigen-presenting cells is B7, which binds the T-cell protein CD28. Binding of the T-cell receptor (TCR) and its co-receptor CD4 to the peptide~MHC class II complex delivers a signal (signal 1) that can only induce clonal expansion of T cells when the co-stimulatory signal (signal 2) is given by binding of CD28 to B7.*

Figure 12: *Regions in Europe covered by a policy of universal immunization (adapted from The Viral Hepatitis Prevention Board-Facts Sheet-1996, [127]).*

Figure 13: *Impact of immunisation strategies against hepatitis B: estimated proportions of cases of hepatitis B prevented based on various immunization strategies (adapted from Margolis H, et al., 1991, [129]).*

Figure 15: Schematic and highly simplified representation of the human immune response to HBsAg.

Figure 14: *Schematic presentation of the molecular basis of the HBV vaccine escape mutant. Anti-HBs made in response to a wild-type HBsAg vaccine recognizes and neutralizes wild-type HBsAg but not mutant HBsAg. Therefore, the HBsAg mutant can escape anti-HBs and establish a productive HBV infection despite successful vaccination against HBV (adapted from Blum HE, 1995, [288]).*