Hepatitis B from diagnosis towards prophylactic single domain antibodies

Hepatitis B van diagnose tot profylactisch enkel-domein antilichamen

(met een samenvatting in het Nederlands)

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Cover: Art impression of the “a”-determinant of HBV complexed with a VHH. (MvR2011)

Contents

Chapter 1: Introduction

Part A: General introduction of the biology of hepatitis B virus

Hepadnaviridae

Hepatitis B virus (HBV) is the prototype member of the Hepadnaviridae family, typed as DNA viruses with high affinity for liver cells. Besides in infected human liver cells HBV DNA can be detected in other organs as kidney, pancreas and in mononuclear cells. Hepadnaviridae are found in other primates and in avian species as ground squirrels, woodchucks, herons and ducks. All viruses within these animal groups infect mainly liver cells and the virions contain (half) double stranded DNA, surrounded by a core protein and envelop protein embedded in lipids derived from the infected host. Another typical feature is that all Hepadnaviridae shed abundant empty surface antigen particles from the liver (22, 23).

Hepatitis B virus infection

HBV infection is possible via contaminated needles, unsafe sexual behaviour, mother-to-child transmission or through blood transfusions of infected blood units. After infection HBV can lead to a chronic infection if the virus is not cleared within 6 months after infection, or to an acute infection, when the virus is cleared within this period. In adults, HBV infections in 5-10% of the cases results in chronic disease. Infections earlier in life lead more often to chronic infections till almost 100% when infected at birth. Also male patients are more likely to develop a chronic HBV infection. As a result of long-term infection the liver is damaged with cirrhosis (22, 23). Additionally, chronically infected subjects have a 100 fold higher risk on hepatocellular carcinoma than none carriers (103). As a result of the long lasting presence of HBV in man, the infection is a global health problem with more than 350 million infected people (42). To reduce the total number of infected subjects a worldwide vaccination program was initiated in the mid-80s and is still on-going (103). In addition the blood supply is tested on the presence of HBV and infectious units are discarded to prevent viral spread in the recipients.

The time course of a typical acute infection is illustrated in figure 1. In general between 2 - 3 months after infection HBV DNA, hepatitis B surface antigen (HBsAg) and Hepatitis B e Antigen (HBeAg) becomes detectable, soon followed by antibodies against the core antigen (anti-HBc). The anti-HBc response is first of the IgM class and later of IgG. The HBeAg is a secreted “core” antigen that shares 90% of the sequence of the core antigen. When the HBV infection is cleared (acute infection) the antigen levels decline and the corresponding anti-HBs and anti-HBe antibody levels become detectable. In chronic infections HBsAg and HBV DNA

remain detectable and depending on the patient status, active or inactive carrier, HBeAg levels decline and anti-HBe is detectable. In chronic active carriers the HBeAg levels remain usually high, accompanied with higher levels of HBV DNA, while in some chronic carriers low levels of HBV DNA are found in conjunction with absence of HBeAg. The absence of HBeAg is therefore more favourable in chronic infections.

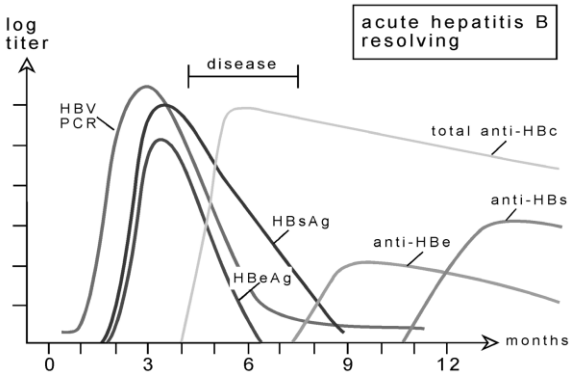

Figure 1 Serological profile of acute hepatitis B virus infection with recovery. Anti-HBs: HBsAg antibody; anti-HBc: Hepatitis B core antigen; HBe: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen by Gerlich personal communication.

Serological Diagnosis of HBV Infection

Diagnosis of HBV is possible by detection of early and constant HBV specific markers present in infected patients. The first marker discovered related to HBV infections was HBsAg, at that time called the Australia antigen. The immunological detection of HBsAg is possible 1-3months after onset of infection and dependent on the course of the infection for 3-6 months during acute HBV infections or longer in chronically infected patients. Advances in molecular biology techniques led to the development of hybridization and polymerase chain reaction (PCR) assays for direct determination of hepatitis B virus DNA (HBV DNA) and made it possible to detect HBV even earlier after infection and in patients with extremely low levels of HBV replication (26). Detection of serum HBV DNA is the optimal method of establishing hepatitis B viremia and is valuable for assessing liver disease activity, decision to receive antiviral therapy, determination of the response to antiviral treatment, and detecting the emergence of drug-resistant mutants. In addition, infectivity of a given individual can also be estimated by the quantification of serum HBV DNA levels. Hepatitis B core antigen is an intracellular antigen that is not detectable in serum. Its antibody, anti-HBc, indicates a prior exposure to HBV, irrespective of the current HBsAg status. IgM anti-HBc is the first antibody detectable in acute HBV infection, which is usually detected within 1 month after appearance of HBsAg. The presence of IgM anti-HBc with high index value usually

indicates a recent HBV infection and this antibody usually disappears within 6 months (35). The combination of these markers sometimes extended with the detection of HBeAg or its corresponding antibodies (anti-HBe) are used to establish the patients' status related to the HBV infection and to monitor the progress of disease together with changes in liver enzymes levels (3).

Genomic organisation

The hepatitis B virus has a compact genomic organization with several overlapping genes (figure 2). In total four genes are present, the longest being p-gene that encodes for the polymerase protein needed for the reverse transcription of the pregenomic RNA of HBV. The c-gene encodes for two proteins that have identical c-terminus, first of all the shorter version which is the core protein that encapsulates the HBV pregenomic RNA and secondly HBeAg, for which the translation starts at the preC start codon. After translation HBeAg is secreted from infected liver cells and thought to enhance tolerance during a HBV infection. The X-gene coding for the X protein seems to be involved in transformation of the hepatocytes or immune regulatory effects that normally lead to apoptosis of the infected cell. Various functions have been designated to the X protein, but for none of the functions undisputed evidence exists. The last gene, named s-gene encodes for three proteins with identical c-terminus, called large, middle and small s-protein encoded by two mRNA molecules: one for the large s-protein and one for both the middle and small s-protein (73, 102). The three proteins together form the outer shell of the HBV particles (22, 23). The large s-protein is known to be involved in viral entry while the middle s-protein seems the dispensable, but together with the small s-protein forms the building blocks for the virion and other HBV related particles (69).

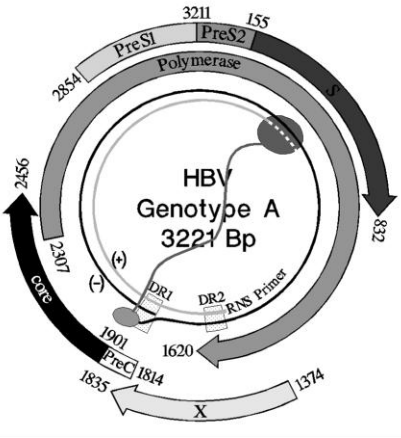

Figure 2: Genomic organization of HBV. Different arrows indicate the different genes on the genome by Gerlich personal communication.

Part B: Hepatitis B replication and infection systems

HBV life cycle

Infection with HBV starts with binding of at least PreS1 to a still unidentified receptor, which is not yet discovered mainly due to the lack of a suitable infection system. However, the study of duck hepatitis B virus (DHBV), which provides a primary cell culture infection system, gave us insight into possible viral-entry mechanisms by the identification of PreS1 attachment to the receptor, carboxypeptidase D (CPD) (7, 94). CPD is a Golgi resident protein cycling between the plasma membrane and the Golgi network (94). Following receptor attachment, DHBV has been shown, through the use of energy-depleting agents, to take the endocytic route of cellular uptake (43). Once DHBV is endocytosed, the virus is targeted to the late endosome, where the large S-protein (L protein) undergoes a conformational change facilitated by the low pH and deforming conditions of this compartment. The conformational change exposes the fusion peptide (TM1) and allows it to anchor and destabilize the endosome lipid bilayer (13).

For HBV there is still little known about how the receptor mechanism works and the entry process leading to infection. Many researchers who tried to isolate HBV binding components from the plasma membrane of either primary human hepatocytes or established cell lines, failed as none of these potential HBV-binding factors seems to have an essential role in HBV entry (27). In vivo HBV infects only humans and some primates, until now almost no easy small animal system has been available (50). The last few years a variety of animal species have been examined for their permissiveness for HBV. Currently the most valuable small animal model for the study of viral hepatitis is the uPA-SCID mouse model (32, 59, 74).

For many years cultures of primary human hepatocytes (PHH) were used that were obtained by immediate perfusion of liver pieces after surgical resection. But this system has a low efficiency of HBV infection and negligible virus shedding (27). Since the observation that primary hepatocyte cultures from Tupaia belangeri (PTH) are susceptible to HBV, a new HBV-susceptible cell line was established, this by showing that HBV infection of PTH is homologous to infection of PHH (28, 29, 44, 50, 98). In order to be independent of primary hepatocyte cultures, the use of human hepatoma cell lines was explored extensively. An often used human hepatoma cell line is the HepG2 cells. These cells have some features of differentiated liver parenchymal cells. Different results were obtained using the HepG2 cells, like successful production of virions after stable or transient transfection of HBV DNA (2, 83, 90). There are also studies that showed binding and uptake of HBV by HepG2 cells, but apparently did not yield a productive infection.

Leenders et al. were the first to show binding of recombinant S surface protein to human hepatocytes, confirmed by De Bruin et al., who also demonstrated internalization of gold labelled subviral particles (18, 49). Qiao and colleagues showed adsorption and penetration of HBV in HepG2 cells (72). Treichel and colleagues showed binding of HBV particles to the human asialoglycoprotein receptor and uptake in HepG2 cells, but like Qiao et al. could not demonstrate infection. (95, 96) Some studies reported the detection of viral antigens upon infection of HepG2 cells that were cultivated in the presence of dexamethosone, insulin or dimethyl sulfoxide (DMSO), but these results have never been reproduced by others (5, 66). Recently Leistner et al. suggested that HBV is initially trapped in the space of Dissé by heparan sulphate proteoglycans and then binds to an unknown high-affinity receptor that confers uptake in a yet unknown department. This based on specifically blockage of HBV infection by pre-incubation of purified virus with heparin or treatment of PTH and HepaRG cells with heparinase (50).

The entry and replication of HBV is shown in figure 3. It is believed that after internalization the virus enters the (late) endosomal compartment where the outer shell, composed of HBsAg is degraded thereby exposing special membrane permeable sequences in the s-protein and in the process passes the core with HBV DNA to the cytoplasm (89). The core particle with nuclear localization signals, binds to the nuclear pore complexes and is transported over the nucleus membrane via nuclear transport factors called importins (40, 41, 80). Inside the nucleus the HBV DNA is repaired or converted to covalently closed circular DNA (cccDNA) that serves as template for the transcription of the different HBV related mRNAs. The viral RNAs include pregenomic RNA (pgRNA), which serves as the template for reverse transcription (81). Newly formed mRNA molecules are transported to the cytoplasm and serve as template for protein synthesis. When a sufficient amount of core protein is formed, pgRNA is encapsulated together with the viral polymerase and reverse transcription is initiated. Completion of the first strand synthesis signals phosphorylation of the core protein and the binding to the nucleus or to the preS1 region of the large s-protein accumulated on the endoplasmic reticulum (ER) (22, 23, 40). Binding of the phosphorylated core particle to the nucleus triggers core particle entry in the nucleus where the core particle is degraded and cccDNA is formed, finally resulting in on average 30-50 cccDNA genomes per infected liver cell depending on disease state (108). Alternatively when sufficient large, middle and small s-proteins are accumulated on the surface of the ER, the core particle binds to the PreS1 part of the s-protein and is secreted through the Golgi system. During the secretion process the 2nd strand synthesis is started but stops most likely due to lack of encapsulated substrates, resulting in progeny viruses with partly double stranded DNA inside. Aside from secreted viral particles viral particles with a size of 42nm, “empty” 22nm particles and filaments are secreted via a different route through the Golgi system. The

efficient translation of the small and middle s-protein results in secretion of at least 100 times more 22nm particles compared to infectious virus particles (22, 23).

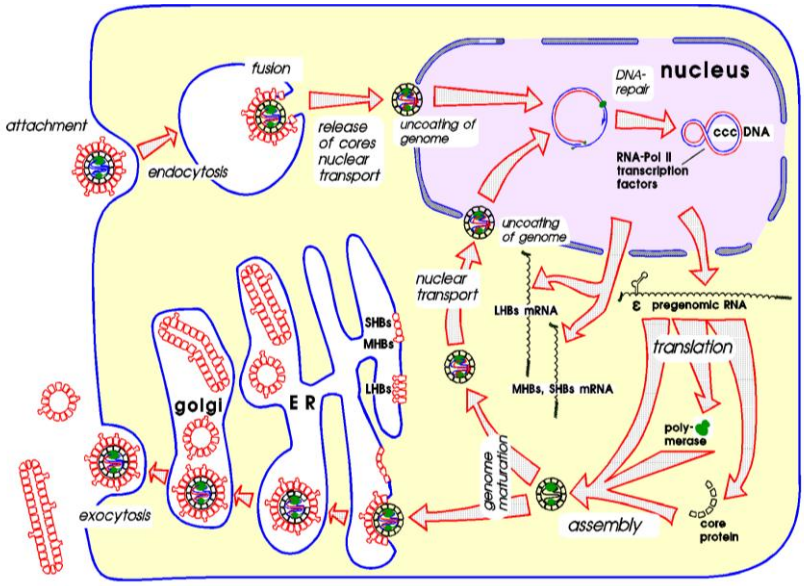

Figure 3: life cycle of HBV. Infection with HBV starts with uptake of the virus and degradation of the outer shell. The free core binds to the cell's nucleus and releases the viral genome. Transcription of the viral mRNA initiates synthesis of viral proteins and the release of progeny virions by Gerlich personal communication.

Hepatitis Delta (HDV)

Rizzetto and colleagues discovered HDV in 1977 by investigating a group of patients with HBV who had severe hepatitis. They showed a novel antigen (delta-antigen) and corresponding antibodies (delta-antibody). The researchers noted that the delta antigen occurred only in HBV patients suffering from severe liver disease (36, 75). The disease was associated with a particle consisting of an RNA genome of low molecular weight encapsulated by HBV envelope proteins (36, 76). This particle was termed the delta agent, or HDV, and was classified under the *Deltavirus* genus. HDV is defective and requires co-infection with HBV for its replication and therefore considered as a satellite virus of HBV. The outer surface of HDV is composed of cellular lipids and the three s-proteins derived from HBV. The particle surface of HDV is predominantly built with the small s-protein with only a few middle and large s-proteins, which is more similar to empty HBsAg particles than infectious HBV particles (45). The HDV receptor on the human hepatocyte remains unidentified, but is thought to be the same as that of HBV because of the shared identity of their outer coat. HDV infectivity is like that of HBV dependent upon a receptor-binding domain in the N-terminal region of the pre-S1 moiety of large HBsAg. A second infectivity region in the antigenic loop of all three envelope

proteins is also required for infectivity, but whether the antigenic loop and the pre-S1 determinants act synergistically or independently in viral entry is unclear (1, 38, 77, 91). The ability of HDV to infected liver cells equal to HBV makes HDV a suitable but less dangerous candidate to study the early steps of HBV and HDV infections.

Part C: Different forms of HBsAg; implications for diagnosis and disease outcome

Large quantities of HBsAg particles are secreted into the bloodstream of patients infected with hepatitis B virus. HBsAg is found in three types of particles: the infectious 42nm spherical Dane particles, the filamentous particles (22x100-200nm) and empty particles (22nm), the latter being the most abundant of these three in human plasma (17, 33). The HBsAg particle is a complex macromolecular structure comprised of lipids and (glyco-) proteins (24, 25, 31, 33).The 22nm HBsAg particles are expressed successfully in and secreted from different cell types like COS, CHO, HepG2, HuH7, Hela and C127 (48, 52, 53, 97, 106). Transfection of the small/middle s-gene results in efficient secretion of 22nm particles with similar appearance compared to serum derived particles under standard EM conditions (2, 48, 56, 101). Analysis of the lipids found in HBsAg particles expressed in COS, C127 or serum derived HBsAg showed similar relative amounts of protein and lipids (19, 25) and confirms the observations from Gavilenes and colleagues (24, 30), who found a protein lipid ratio of about 70% in HBsAg purified from sera. All HBsAg expressed by the different cell systems contain the different forms of small and middle s-proteins, depending on the gene used for expression, when analyzed on SDS-PAGE. The small s-protein is found in two forms, non-glycosylated (P24) and glycosylated (GP27), the middle s-protein as a monoglycosylated (GP33) and diglycosylated (GP36) version and the large s-protein in non-glycosylated (P39) and glycosylated (GP42) form. All three proteins share a common C-terminal part and are N-terminally extended with a PreS2 region in the case of the middle s-protein or with a PreS1 and PreS2 region in the case of large s-protein (33, 60, 93). All HBsAg particles are composed of the small and middle s-protein, while the large s-protein is almost exclusively found in the filaments and Dane particles. Analysis of the particles on density gradients as CsCl showed that the major HBsAg activity is found at a density of 1.2 g/ml (24, 48). The steps of the biosynthesis of the 22nm particle biosynthesis have intensively been studied and gave insight in the different steps of the 22nm particle formation. After translation, the S-protein is inserted into the membrane of the endoplasmic reticulum (ER) where it is arrested to form dimers in the presence of protein disulfide isomerase (PDI) (37, 67). After accumulation, about 48 dimer S-proteins are directed to the early Golgi (the intermediate compartment) where the S-proteins aggregate to form 22nm particles (37)'(82, 85). Here some of the 16 cysteins residues present in the S-protein crosslink to form a highly stable 22nm particle

(31, 37). The budding is only poorly understood and involves substantial reorganization of the membrane lipids with help of chaperones (24, 25, 37, 78). After budding, the 22nm particle leaves the cell via the constitutive pathway of secretion. The S-protein contains three hydrophobic domains located at amino acids (aa) positions 11 to 28, 80 to 98, and 169 to 226 (31, 31, 67, 87). The domains separate two hydrophilic regions aa29 to aa79 and aa99 to aa168 (figure 4), with the latter being referred as the HBs antigenic region or.

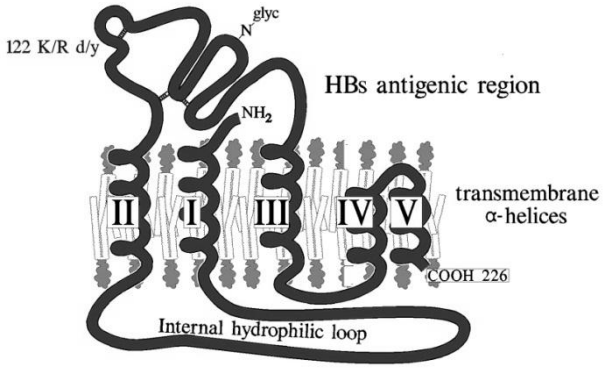

Figure 4: Proposed topology of the envelop protein by Stirk (88), adapted by Gerlich personal communication. The predicted hydrophobic domains I-V are indicated.

Analysis of mutated and deleted forms of the S-protein have shown that both the entire first and second hydrophobic domains serve an essential function in determining protein topology (8, 21, 57, 70). The first region mediates targeting to the ER membrane, despite lacking an N-terminal cleavage signal sequence (8, 21). The second domain anchors the protein in the lipid bilayer (8, 21). This results in a topology where the first hydrophilic region has a cytoplasmic orientation and the second hydrophilic region is oriented to the lumen (i.e. external in the mature particle). The cytoplasmic orientation of the first loop is confirmed by the observation that the sequence is accessible to proteases when anchored in the ER and is inaccessible by proteases in the mature particle (20, 21, 31, 87). Furthermore, a potential glycosylation site (NXS/T) at amino acid 59 in the internal hydrophilic loop, present in various subtypes of HBV is left unused. The lumenal orientation of the second hydrophilic region is confirmed by the observation that the sequence is glycosylated and the region is accessible to the immune system (12, 54, 62). Currently there is no consensus on the confirmation of the last hydrophobic region (aa169 to 226; IV,V; figure 4), which is suggested to pass the lipid membrane twice as found by computer aided modelling either or not combined with proteinase K proteolysis studies (31, 39, 54, 68, 86, 88).

HBsAg subtypes

Over time HBV has evolved in different serological groups, based on the reactivity of the hepatitis B surface antigen (HBsAg) with anti-sera (4, 16, 47). Initially four subtypes were recognized and denoted as Adw, Ayw, Ayr and Adr (4, 47), but this number was soon expanded to in total 9 different subtypes after testing of large number of patient sera (16). Early studies showed that these 9 subtypes were restricted each to different parts of the world (16) and could later be linked to the 8 genotypes, A-H (table 1) (58, 61–63, 65). Variants belonging to one genotype differ less than 4% to each other, while compared to other genotypes more than 4% sequence difference is observed.

Table 1: Geographical distribution of HBV genotypes and subtypes over the world adapted from Couroucé et al.

Genotype	HBsAg	Geographical distribution
A	Adw 2	Europe, North America, Africa
	Ayw 1	Africa
B	Adw 2	Far East
	Ayw 1	Far East
C	Adrq-	Pacific
	Adr/Ayr	Far East
	Adw	Japan, Indonesia
	Ayr	Far East, Pacific
D	Ayw 4	United States
	Ayw 2/Ayw 3	Worldwide
E	Ayw 4	Africa
F	Adw 2	South America
	Adw 4	Polynesia, Alaska, Central and South America
	Ayw 4	South America
G	Adw2	USA
		France
H	Adw4	Mexico, California
	Ayw4	Nicaragua

Comparison of the sequence data of the nine subtypes revealed that the d/y determinant was related to the presence of a lysine (“d” determinant) or arginine (“y” determinant) at amino acid 122. The w/r determinant was related to the presence of the same positively charged residues at position 160, i.e. lysine for the w determinant and arginine for the r determinant (62, 63, 65). Other sub determinants were also related to amino acid changes, but in those cases more than one amino acid positions seemed to be involved (61–63) (figure 4).

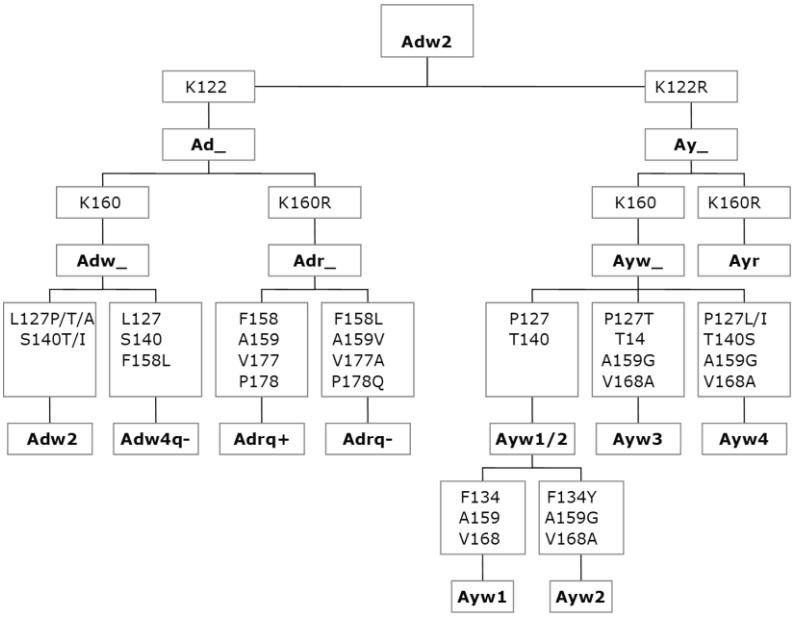

Figure 4: schematic depiction of most frequently found HBsAg subypes, indicted are the mutations that are involved in type switching. (64)

HBsAg mutants

The first description of a naturally occurring HBsAg mutant was made in the breakthrough infection of a child born to a HBV-positive mother (107). The virus was vertically transmitted despite the child being vaccinated and passively immunized against HBV. Sequencing of the isolated virus revealed a single amino acid substitution at position G145R of the small s-protein (11) and remained unchanged even after 10 years after infection. The single amino acid change in the immune dominant region has such a dramatic impact on the conformation that it renders antibodies unable to neutralize this virus (84). Shortly after discovery of the G145R mutant a wide range of mutants was described, including many amino acid substitution mutants across the "a"-determinant, amino acid insertions into the "a"-determinant, and deletion mutants (10, 34, 99, 105). Important to the healthcare management of HBV infection is detection of HBsAg mutants by diagnostic assays. HBsAg is a sentinel marker in blood bank donor screening to prevent transmission of HBV infection in patients receiving transfusions. Due to the restricted reactivity spectrum of most monoclonal antibodies most diagnostic assays use mixtures of antibodies to ensure detection of all known HBsAg variants (46, 55). The detection spectrum of the diagnostic assay is determined with sera from patients infected

with subtypes found throughout the world. The panel from Couroucé is often used for this purpose. Discovery of less frequent HBsAg mutants, like the G145R mutant, expanded the initial used panel with samples from patients infected with HBsAg variants containing these mutations (104).

Treatment of hepatitis B infections

Today the (PEG-)Interferon (IFN) is the first choice for the treatment of HBV infections, but is only effective in 30% of the patients. Major disadvantages of (PEG-)IFN therapy are the subcutaneous administration and frequent side effects. Particularly flu-like symptoms, cytopenia and psychiatric adverse events are frequently observed, but outweighs by the high rate of sustained response (15). In the last decade there has been a major advance in the treatment of chronic hepatitis B with nucleos(t)ide analogues. The nucleos(t)ide analogues can be used in case IFN therapy fails (9). Treatment with these antiviral agents inhibits the viral polymerase activity and thereby viral replication. Advantages of nucleos(t)ide analogues are the oral administration, rapid decline in HBV DNA and minimal side effects. A major disadvantage is that the majority of patients need prolonged or even indefinite therapy, as sustainability of response after discontinuation of therapy is limited. The first licenced antiviral drug was Lamivudine or 3TC, but its use was limited due to drug selected specific polymerase mutants (6, 51). New antiviral drugs soon followed with less frequent occurrence of mutants during long term use. Examples of licenced drugs are: Adefovir, Entecavir and Telbivudine. The different drugs can be used after failure of lamivudine treatment or in combination (15). Today treatment is not successful for all HBV infected patients and welcomes new drugs that can change the patients' infection state or even cure the patient from the HBV infection.

Part D: Studies conducted for this thesis

Diagnosis of HBV is important for treatment and to prevent further spread of the virus. Today Hepatitis B surface antigen (HBsAg) is the key marker for screening and laboratory diagnosis of HBV infection and the first serological marker to appear during the course of HBV infection. The sensitivity of measuring HBsAg in serum samples depends on the detection threshold of immunoassays. The lower the detection limit for HBsAg, the smaller the diagnostic 'window phase' in early infection (79) is and the higher the capability for detecting the smallest amounts of HBsAg in asymptomatic patients and chronic carriers (10). Due to of the genetic diversity of HBV, sensitivity of HBsAg assays is also dependent on antigenic variation of HBsAg. In fact, some HBsAg mutants that emerge after selection by immune pressure can escape detection by some commercial HBsAg assays (11, 14, 71, 100). This thesis aims at selection of antibody pairs suited for sensitive detection of HBsAg and to provide evidence in direct comparison to

other HBsAg assays available.
In Chapter 2 the ability of different monoclonal antibodies to detect different groups of HBsAg mutants is described and paved the way for the selection of antibody pairs enabling detection of HBsAg mutants. The initial solution for the detection of all known HBsAg mutants included the use of an anti-PreS2 antibody that recognized a linear epitope in the middle s-protein present in all HBsAg particles. The Hepanostika HBsAg Plus was shown to detect all HBsAg mutants but not pursued due to specificity problems (i.e. negative samples were detected as positive samples) and the fact that HBsAg variants were found lacking the non-essential preS2 region (60, 92). Meanwhile we identified a unique human monoclonal antibody reactive with HBsAg variants.
Chapter 3 describes the characterization of this antibody using recombinant HBsAg baring single amino acid substitutions.
Knowing the different reactivity spectra of the monoclonal antibodies we could select the best pairs for efficient and sensitive detection of HBsAg as checked with the HBsAg mutants, which is described in Chapter 4, with the clinical performance outlined in Chapter 5. The importance of the developed assay was emphasized in a study comparing 70 HBsAg assays (79). The diagnosis of HBV positive patients enables us to prevent viral spread, but more importantly it initiates an early treatment of the infected patients. Today antiviral treatment is started when the liver of the infected patients are compromised, mainly due to the fact that HBV clearance is hard to accomplish. A low replicative state with little to no liver damage is therefore a practical end point for treatment (3, 9). Alternative therapies are thus still a welcome addition to the already available antiviral repertoire. The knowledge obtained in the first part of the thesis made us decide to characterize selected VHH antibodies against HBsAg. In chapter 6 the characterization of the neutralizing VHH is summarized. The small size of the VHH and efficient neutralization of HBV by the VHH made us believe that the VHH recognized an important region of the HBsAg particle possibly involved in binding to a secondary to be discovered receptor. The final chapter 7 outlines the outcome of computer modeling studies presenting a possible conformation of the c-terminal region of HBsAg (aa107-226) including the ‘a’-determinant, reflected on the personal observations done with VHH’s, monoclonal antibodies and published data about antibody recognition and site directed mutagenesis.

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Chapter 2: Characterization of the reactivity pattern of murine monoclonal antibodies against wild-type hepatitis B surface antigen to G145R and other naturally occurring “a” loop escape mutations

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The hepatitis B surface antigen (HBsAg) “a” domain harbors major B-cell epitopes. Viruses with mutations in this region emerge after vaccination or during hepatitis B immune globulin (HBIg) prophylaxis. A strain with G145R replacement has been almost invariably isolated as a major escape mutant. We investigated mutant antigen-antibody interactions with direct binding assays. G145R and 16 other naturally occurring recombinant HBsAg mutants were expressed in mammalian Cos-1 cells. The reactivity of a panel of 28 murine anti-hepatitis B surface antigen (anti-HBs) monoclonal antibodies to mutant antigens was measured with enzyme immunoassay and expressed as percentage compared with the wild-type (wt) HBsAg signal for each antibody. All point-mutated proteins displayed diffuse intracellular immunofluorescent labeling corresponding to a secretory pathway. Monoclonal antibodies (mAbs) were classified according to different binding patterns. The effect of mutations on antibody binding differs depending on the amino acid involved and on the location within the “a” loop. As expected, most antibodies had absent or negligible binding (<40%), notably with residue 145 replacements. However, we identified antibodies that reacted with conformational epitopes but nevertheless had adequate reactivity (>40%) with all mutant antigens, including G145R. The effect of G145R was more pronounced than that of G145A. A subgroup of antibodies had substantially increased recognition (>120%) of antigens with mutations in the first loop. We demonstrated that antibodies can be selected or combined that react with all mutants investigated, including G145R. These data offer perspectives for improving anti HBs-based protection against hepatitis B**.**

Abbreviations: HBsAg, hepatitis B surface antigen; anti-HBs, antibody to HBsAg; HBV, hepatitis B virus; aa, amino acid; HBIg, hepatitis B immune globulin; wt: wild type; mAb: monoclonal antibody; PBS, phosphate-buffered saline. The single-letter amino acid code was used.

Chronic hepatitis B continues to be a major cause of morbidity and mortality worldwide (1). Serological diagnosis, monitoring of seroconversion, vaccination, and immunoprophylaxis after liver transplantation all depend on the binding between hepatitis B surface antigen (HBsAg) and anti-HBs antibodies. The hepatitis B virus (HBV) envelope consists basically of viral small (S), middle (M), and large (L) HBsAg, and a host cell-derived lipid membrane (2).

Major B-cell antigenic epitopes reside in the group-specific “a” determinant region, which extends from amino acids (aa) 124 to 147 of the S protein (3). This highly conformational structure consists of two loops held by disulfide bridges (cysteines 124-137 and 138-147 or 149) projecting from the viral surface(3–7). Immunoreactivity depends on spatial arrangements of distinct peptide regions and has been analyzed by competitive binding assays and with synthetic peptides (8). Antibodies to the “a” determinant are thought to confer protection against the major HBV subtypes (adr, adw, ayr, ayw) and are pivotal for HBV clearance, whereas subtype-specific antibodies were not found to be neutralizing (9–11).The “a” loop is the most important target for diagnosis and immunoprophylaxis; high-affinity antibodies against second-loop epitopes appear in responding vaccinees, and are used for hepatitis B immune globulin (HBIg) prophylaxis in liver-graft recipients. Consequently, there is increasing concern about the isolation of mutant viruses with aa substitutions in the “a” loop.

The HBV life cycle includes an intracellular RNA pregenomic intermediate, which is reverse-transcribed within the nucleocapsid. The absence of proofreading ability of RNA-dependent DNA polymerase enables the virus an estimated mutation rate of 2.10^{-4} substitutions per site per year in spite of its overlapping genes (6). HBV variants are distinguished from mutants. Natural sequence variability of HBsAg has led to classification into subtypes. Serological subtype determinants (adw, ayw, adr, ayr) are identified with subtype-specific antibodies (12) and correspond to single aa substitutions. Based on nucleotide sequence data, 6 genotypes have been identified (13). Variants are found in natural isolates and have a geographical distribution. Mutants emerge spontaneously during the course of the infection, as breakthrough infections under immunological pressure by the host, or during treatment with antiviral xenobiotics (14). They facilitate viral persistence and compromise anti-HBs–mediated immunoprotection. Nucleotide point mutations causing various amino acid replacements within the “a” loop have been identified in chronic carriers and among fully vaccinated subjects,(5,15–22) and during prophylaxis of recurrent HBV infection with high-dose HBIg in liver-transplant recipients (23–27).

An increase of breakthrough HBV infections during HBIg therapy associated with increased use of recombinant versus plasma-derived antigen to generate HBIg was reported recently(28). In the vast majority, an arginine-for-glycine replacement at residue 145 was identified as the prevailing viral form. This G145R mutant is a stable strain that can be transmitted directly and can cause liver disease (17–19,29,30). S mutant strains may even be more pathogenic than wild type (wt), because severe hepatitis and elevated transaminases were more frequently seen after their emergence (31,32). Recently, a less favorable outcome with respect to liver-graft survival post transplantation was associated with the isolation of G145R mutant virus (28).

It is assumed that mutations alter the immunological characteristics of the S protein, although there are no data about their reactivity with a panel of monoclonal antibodies (mAbs) that is large enough to identify a spectrum of different reactivity characteristics. Virtually identical lipoprotein particles are produced upon transfection of cultured cells with vectors encoding HBsAg, and this allows the analysis of antigenicity of mutated proteins obtained with site-directed mutagenesis (4). We therefore investigated direct antibody binding to various naturally occurring single and double HBsAg variants and mutants that cover the “a” loop by expressing cloned HBsAg in a mammalian cell line and testing the translated proteins for reactivity with a panel of mAbs directed against purified native wt HBsAg.

MATERIALS AND METHODS

Oligonucleotide-mediated site-directed mutagenesis and recombinant HBsAg mutants.

A DNA fragment of 900 bp corresponding to middle HBsAg, subtype ayw3, was used for the construction of several point-mutated DNA sequences with primer site-directed mutagenesis (Table 1). We obtained single and double point-mutated HBsAg proteins with the following aa replacements (Table 1): R122K, A128V, Q129R, G130N, M133T, Q129R and M133T, Y134F, S136Y, K141W, D144H, G145R, G145A, Q129R and G145A, Q129R and G145R, T148A, S154E, K160R (5,10,12,15–17,22,23,25,26,30,31,33–46); EMBL Data Library accession no’s. S41870 and S36654). Double mutations were included because the same mutation can induce different changes in predicted antigenicity depending on the rest of the sequence. For the construction of an “a” knock-out mutant, sequence aa 120-160 comprising the “a” loop was replaced by aa 30-79 of small HBsAg.

Table 1: Nucleotide replacements within the small HBsAg genome sequence obtained with oligonucleotide mediated site-directed mutagenesis, and corresponding amino acid single point mutations

Nucleotide Number*	Nucleotide Substitution	Amino Acid of S HBsAg	Amino Acid Mutation	Phenotype (subtype)	References
571, 572	CGG→AAG	122	R→K	d→y	10
590	GCT→GTT	128	A→V		25, 33, 34
593	CAA→CGA	129	Q→R		5, 15, 31, 33, 35
595-597	GGA→AAC	130	G→N		33, 35, 36
605	ATG→ACG	133	M→T		22, 26, 37
608	TAT→TTT	134	Y→F	d→y	12, 38
614	TCC→TAC	136	S→Y		34
628-630	AAA→TGG	141†	K→W		
637	GAC→CAC	144†	D→H		accession no: S41874
641	GGA→GCA	145†	G→A		15, 26, 31, 39, 40
640	GGA→CGA	145†	G→R		5, 16, 17, 23, 30, 41-44
649	ACC→GCC	148†	T→A		45
667,668	TCA→GAA	154	S→E		accession no: S36654
686	AAA→AGA	160	K→R	w→r	10, 34

*Nucleotide numbers starting from the unique EcoR1 site of HBV (serotype ayw3).
†Amino acids within the second loop of the ‘‘a’’ determinant (aa 139-147[149]).
S41874 (Lai ME, et al., submitted to the EMBL Data Library, Jan. 1994).
S36654 (Chirara M., submitted to the EMBL Data Library, Nov. 1992).

Construction of the Eukaryotic Expression Vector pSG8puro With HBsAg and Transfection of Cos-1 Cells.

Mutant constructs in plasmid pSP70 were digested with SmaI. The HBsAg fragments were isolated from agarose gel with the Gene-CleanII kit (BIO 101, Carlsbad, CA) and ligated into the blunt-ended mammalian expression vector pSG8puro at the EcoRI site under the control of the SV40 promoter. HBsAg subtype ayw3 was used as wt and the correct orientation was verified with restriction fragment analysis using Xho1. Cos-1 cells were chosen because of high expression levels, secretion into the medium, and glycosylation of translated products; $1.5 * 10^6$ cells were transiently transfected with 10 µg plasmid DNA in 0.2 mL phosphate-buffered saline (PBS) in a 4-mm cuvette at 300 V and 125 µF using a BIORAD electroporator. Cells were cultured for 3 days in Dulbecco’s modified Eagle medium containing 10% fetal calf serum, 2 mmol/L glutamine, 50 µg/mL gentamycine, and nonessential amino acids. Cells were harvested after 3 days of culture and lysed in lysis buffer (50 nmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 100 µg/mL phenylmethylsulfonyl fluoride). Expression was controlled with Western blotting using murine mAb HBS.OT43 directed against a linear sequence in the PreS2 domain and quantified using the Hepanostika HBsAg Uni-Form II plus (Organon Teknika, Boxtel, the Netherlands). Samples were diluted in normal human serum.

Preparation of Murine Monoclonal Antibodies.

All monoclonal anti-HBs antibodies were produced by Organon Teknika according to standard methods. Pepsin-treated or native wt HBsAg prepared from pooled plasma was used as antigen.

Immunofluorescence Assay.

Transfected Cos-1 cells were cultured for 3 days on poly-L-lysine–coated glass slides and then fixed with 3% paraformaldehyde in PBS. After washing with PBS, cells were incubated with mAb HBS.OT43 (1:500; incubation buffer 1% gelatine, 0.1% Triton X-100, 0.25% bovine serum albumin, 1% normal swine serum in PBS) for 1 hour. Cells were then washed and incubated with fluorescein isothiocyanate–conjugated rabbit anti-mouse IgG (1:100) in incubation buffer for 30 minutes. After dehydration, cells were embedded in embedding fluid MOWIOL, and examined with fluorescence microscopy for expression and trafficking pattern of HBsAg.

Immunoscreening of Mutant HBsAg Proteins.

Sheep anti-mouse immune globulins were coated on the solid phase of 96-well micro titer plates. Murine anti-HBs mAbs (Fig. 1) were added to the solid phase at a 1:1,000 dilution. Normal goat serum (20%) including Triton X 100/PBS (1%) was used as diluent. After incubation for 1 hour at 37°C, extensive washing with PBS-Tween was performed. Subsequently, each recombinant HBsAg was diluted to a preset concentration (U/mL) at which wt HBsAg gave an absorption signal of 1,000 at A450 nm with that mAb (Fig. 1). After incubation for 1 hour at 37°C and extensive washing, sheep anti-HBs horseradish peroxidase conjugate was added, followed by incubation for 1 hour at 37°C. Tetramethylbenzidine-peroxide substrate was then added and followed by incubation for 30 minutes at ambient temperature. For each mAb, the reaction was stopped using 1 mol/L sulfuric acid, and reactivity was measured by spectrophotometry in an automated plate reader at 450 nm. For each mAb, culture medium of nontransfected Cos-1 cells was included as negative control. The reaction obtained with wt HBsAg was set at 100%. The signal obtained for each individual mutant at the preset concentration was compared with the wt signal and expressed as percent change of reactivity. Results were obtained as the means of 3 experiments. Reactivity was expressed numerically with 1 = <40% (absent or negligible); 2 = 40%-80% (moderate); 3 = 80%-120% (comparable with reaction to wt); 4 = 120%-160% (increased); and 5 = .160% (strong) (Fig. 1).

	HBS.OT11	HBS.OT24	HBS.OT33	HBS.OT39	HBS.OT31	HBS.OT40	HBS.OT13	HBS.OT30	HBS.OT29	HBS.OT28	HBS.OT18	HBS.OT14	HBS.OT32	HBS.OT3	HBS.OT26	HBS.OT25	HBS.OT42	HBS.OT41	HBS.OT4	HBS.OT27	HBS.OT16	HBS.OT19	HBS.OT5	HBS.OT2	HBS.OT35	HBS.OT34	HBS.OT9	HBS.OT17
122 R/K	3	3	2	2	2	2	3	2	3	2	3	3	2	2	3	1	3	1	1	3	5	5	3	5	2	1	2	1
128 A/V	3	3	3	2	3	3	3	3	3	3	3	3	2	3	2	2	2	2	2	5	4	4	4	4	4	3	3	3
129 Q/R	3	3	4	4	3	3	3	3	3	4	3	3	2	2	3	3	3	3	2	5	5	4	5	4	5	5	4	3
130 G/N	2	2	2	1	2	2	2	2	2	2	1	1	1	1	1	2	1	1	1	1	2	1	1	1	2	2	2	2
133 M/T	3	2	2	3	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2	2	2	1	2	3	3	3	2	3
134 Y/F	2	3	3	3	3	2	2	3	3	3	2	2	2	2	2	2	2	2	2	3	4	3	4	3	3	2	3	2
136 S/T	2	2	2	1	2	2	2	2	2	2	2	3	2	2	2	2	3	1	1	2	1	1	1	1	1	1	1	3
141 K/W	3	2	2	3	3	2	2	2	2	2	3	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	2
144 D/H	3	3	2	3	3	2	2	2	2	1	1	2	1	1	1	1	1	2	1	1	3	2	1	4	4	4	1	3
145 G/A	3	3	2	3	3	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	3	5
145 G/R	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	4
148 T/A	2	1	1	1	1	2	2	2	3	2	3	4	2	3	3	3	3	2	2	3	1	1	1	2	1	1	1	3
154 S/E	2	2	2	2	2	2	3	3	3	2	3	3	2	2	3	2	2	2	2	2	1	2	2	3	2	2	2	3
160 K/R	2	2	3	2	3	2	2	3	2	2	3	3	2	2	2	3	2	2	2	3	3	2	2	3	2	2	2	3
129/133	3	3	3	3	3	3	2	2	3	3	2	2	2	2	2	2	2	3	2	5	4	3	5	3	5	5	4	3
129/145A	3	3	3	3	3	2	2	2	2	3	1	1	1	1	1	1	1	2	1	1	2	1	1	3	4	4	4	3
129/145R	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	5	3	3	3
HBsAg wt U/ml	0.1	0.1	0.12	0.59	0.11	0.11	0.08	0.09	0.07	0.18	0.1	0.34	0.47	0.17	0.16	0.13	0.16	0.13	0.13	0.79	0.39	0.79	0.52	0.67	0.67	0.59	0.17	0.21

Fig. 1. Immunoscreening of a panel of anti-HBs mAbs with single and double point mutations in the HBsAg "a" determinant region. The reactivity of each mAb was compared with the absorbance signal obtained with wt HBsAg and numerically expressed as 1 = <40%; 2 = 40%-80%; 3 = 80%-120%; 4 = 120%-160%; and 5 =.160% change. Bottom row: concentration (U/mL) of each mAb needed to obtain a preset absorption signal with wt HBsAg of 1,000 by spectrophotometry at 450 nm.

RESULTS

Expression of HBsAg Proteins.

All HBsAg constructs expressed in Cos-1 cells were detected in cell lysates with Western blot as 35-kd proteins (data not shown). As expected, the "a" determinant knock-out mutant had a slightly higher molecular weight. With the immunofluorescence assay, a diffuse granular intracellular labeling was seen for wt and all point-mutated HBsAg, as expected for a protein that is translated at the endoplasmic reticulum and is transported to the cell membrane via the Golgi apparatus (Fig. 2A and 2B). Single and double mutations do not affect the efficiency of HBsAg secretion. The "a" determinant knock-out construct, however, was retained as a localized perinuclear aggregate (Fig. 2C). The "a" determinant knock-out construct is thus translated, but not properly transported and secreted.

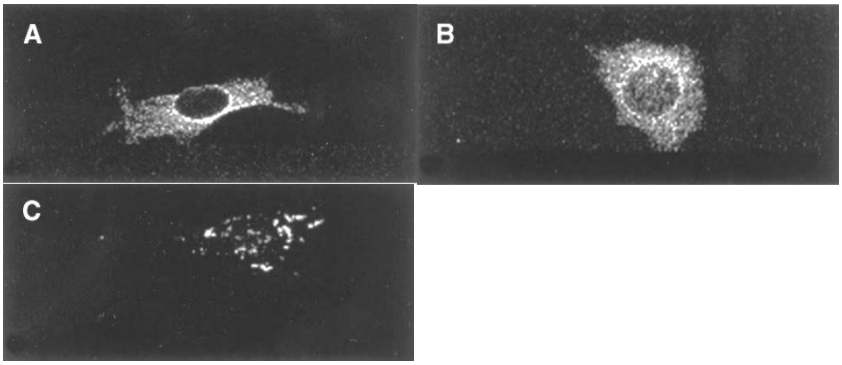

FIG. 2. Immunofluorescent labeling of wt and mutant HBsAg in Cos-1 cells transfected with pSG8puro-HBsAg expression constructs. Cos-1 cells transfected with an expression construct containing wt HBsAg, HBsAg with aa point mutations in the “a” loop, and HBsAg with replacement of the “a” loop (“a” determinant knock-out mutant). Immunofluorescent labeling with mAb HBS.OT43 of wt (A), G145A mutant (B), and “a” determinant knock-out (C) mutant.

Immunoscreening of Mutants.

Immunoscreening was performed on cell culture supernatants of all constructs, except for the “a” determinant knock-out mutant, which was not secreted. Three mAbs were excluded from the study, because no reactivity was observed with any of the antigens. Presumably, they bind outside the region covered by the constructs or recognize a HBsAg subtype different from ayw3. All other antibodies (HBS.OT No.2-5, 9, 11, 13, 14, 16-19, 24-35, 39-42) showed reactivity with the wt HBsAg construct (Fig.1). Every single mAb displayed a specific binding pattern, illustrating the abundance of the antigenic properties of the “a” loop. However, characteristic reactivity patterns shared by several mAbs could be defined. Most antibodies had a virtually absent binding (<40%) to both the R and A mutations at residue 145, albeit to a variable degree. In general, these 145 mutations together with other amino acid replacements located in the second loop, notably at position 141 and 144, had the largest impact on antibody binding. Antibodies whose reactivity with aa 145 single mutations was influenced significantly were also strongly influenced by the corresponding double mutations. In contrast to aa 145 mutations, and to a lesser extent aa 141 and 144 mutations, the changes in reactivity of mAbs with substitutions at the other locations were much less pronounced. In this context, the binding with another frequently observed escape mutant at residue 129 is noteworthy. Interestingly, for some mAbs, we observed an increased reactivity (>120%) in particular with aa 128, 129, and 129/133 replacements. We classified the mAbs into 8 groups based on similar reactivity patterns. The first group consists of 3 mAbs that give the preset signal at low concentrations and have a comparable degree of reactivity to wt and all mutant antigens. In particular, mAb HBS.OT11 adequately bound to all mutants with a relative strength of 2 to 3 (40%-120%), including both the R and A substitutions at

residue 145; the same applies for HBS.OT24 and 33, with the exception of T148A. This T148A mutation is exceedingly rare, however. We assume that these mAbs bind to conformational epitopes, because they do not recognize denatured proteins in Western blots and do not bind to linear 25mere peptides covering small HBsAg (data not shown). This is an important group because it contains high-affinity antibodies that are not sensitive to the mutations tested. The second and the third group both contain 1 mAb. HBS.OT39 is sensitive to 3 mutations (aa 130, 136, and 148), whereas HBS.OT31 does not bind to G145R and T148A. Group 4 (HBS.OT 13, 28-30, 40) is characterized by a lack of reactivity specifically to both single and double mutations with an R replacement at residue 145. Reactivity to all other proteins is adequate, including the 145A mutants. Group 5 is the largest group and contains 9 mAbs (HBS.OT3, 4, 14, 18, 25, 26, 32, 41, 42) that are sensitive to many of the mutations, most notably to those in the second loop, but also to aa 130 replacements. They completely lack reactivity with both aa145 mutations. Peptide mapping studies showed that some of these mAbs bound conformational epitopes (HBS.OT3, 4, 14, 18, 32), while others recognized linear epitopes (HBS.OT25, 26, 41). mAbs in group 6 are characterized by striking quantitative differences in reactivity. Most do not bind to aa 145 and generally to all mutations in this genomic area, but they have a high reactivity (>120%) notably with first-loop mutations including aa 128 and 129. Group 7 (HBS.OT9) does not bind well to most single second-loop mutations, but has strong binding to double mutations including aa 145. Group 8 (HBS.OT17) has an adequate reactivity with all mutants, and should be mentioned separately because of its strong reactivity with both R and A 145 mutations. It has a very low binding to aa 122 replacement, and thus is subtype-specific. This antibody recognizes a linear epitope between aa 106 and 144.

DISCUSSION

The concept of viral immune escape from anti-HBs–mediated immunological pressure of the host is favored by several observations: 1) mutations are selected after the appearance of or during administration of anti-HBs antibodies; 2) they disappear after discontinuation of immunoglobulin therapy, with reversal to wt virus as predominant strain; 3) they do not appear randomly, but mainly in clusters corresponding to antigenic domains; and 4) 80% of nucleotide changes give rise to aa substitutions, implying selection at the protein level (11,13,24,26). Selection of escape mutations in the HBsAg “a” loop has an impact on all aspects of anti-HBs based protection against hepatitis B. The first report of “a” determinant escape mutants was in Italian children from HBV-positive mothers who received vaccination and immunoglobulins. A point mutation at residue 145 changing G to R was found (41). Later, several other point mutations were identified among subjects who acquired HBV breakthrough infections after vaccination with recombinant or plasma-derived vaccine, but the G145R mutant was almost invariably found (15–

17,19,47–49). Hsu et al. isolated 145R and 126A mutants from vaccinated infants born to carrier mothers that persisted during follow-up of 11 years (31). The inoculum transmitted was generally wt strain. In one mother-infant pair, a Q129R substitution was also found in maternal serum, suggesting direct transmission. In spite of the effectiveness of HBIg prophylaxis, liver-graft infection with HBV still occurs in about 30%. HBIg has no selection pressure on the pre-S region, although this domain harbors B and T-cell epitopes (50), but apparently it has this effect on the “a” loop. In one study, mutations were absent in 93% of pre-transplantation clones analyzed, whereas 50% of those who developed recurrent hepatitis B while under HBIg had aa substitutions involving the “a” determinant in post transplantation samples that changed predicted antigenicity (24). There was a correlation with the duration of therapy, and most (78%) reverted back to pre-transplantation sequences after discontinuation of HBIg. The post transplantation emergence of “a” loop mutations has been reported with both polyclonal and monoclonal HBV-specific immunoglobulins (6,23,51), and they were only rarely found in patients without protective anti-HBs titers at the time of graft infection (24,26). In two of three patients with rapidly progressive fibrosing cholestatic hepatitis following graft HBV infection despite HBIg, the G145R mutant was isolated. In one case, this mutant was detected as a subpopulation of, 5% of the viral load before orthotropic liver transplantation (25). HBsAg mutants survive in carriers with a long time course of hepatitis B (21,33,37,42). Zhang et al. found aberrations at 11 of the 69 “a” loop nucleotide positions in 15 anti-HBs – and HBV-DNA–positive patients (33). These mutations occur in HBsAg-positive samples, but they are more frequently detected after anti-HBs seroconversion (12,35). Diagnosis failure has been associated with surface gene mutations, including G145R (39,52). HBsAg missed with polyclonal antibody–based assays had novel or very rare mutants within the central region (14). Because protection against HBV infection correlates to the appearance or administration of anti-HBs antibodies, changed antigenicity of surface mutants affects most clinical aspects of hepatitis B: chronicity, vaccine efficacy, effectiveness of HBIg immunoprophylaxis post transplantation, and diagnostic accuracy. Results from direct binding studies may have consequences for vaccination strategy and for generation of HBIg, which is derived from subjects vaccinated with recombinant HBsAg. There is only limited information on the reactivity of mutant HBsAg with anti-HBs antibodies (7,15,16,46,53). Published data are based on non-mammalian expression systems and a small number of antigens and antibodies. They are none conclusive as to whether which mutants escape antibody binding or whether the escape phenomenon applies to all antibodies that are induced with wt antigen. Bruce et al. expressed mutant HBsAg in yeast and performed radio immunoassay with serum-derived polyclonal anti-HBs; 145 R, K, and E substitutions all had the same level of binding affinity as found for the negative control (7). Waters et al. also found that binding of mAbs with the G145R mutant was abolished (53). Antibodies elicited by administration of the mutant protein to mice did not recognize the native protein

well, supporting the substantial alteration of immunogenicity. Recently, four HBsAg mutant proteins (126S, 129H, 129R, 145R) expressed in yeast were tested with anti-HBs mAb. Various degrees of altered reactivity were observed, with 145R and 129R exhibiting the lowest binding to all antibodies tested (15). We constructed mutant antigens expressed in a mammalian cell line and an extensive panel of 28 mAbs against native wt HBsAg. The antigens included all major mutants described in the literature. The reactivity pattern led to classification of the antibodies into 8 groups. We identified mAbs that reacted with all mutants, including G145R, to a comparable degree as with wt antigen. Some of these mAbs recognized conformational “a” loop epitopes, because they did not bind to corresponding linear peptides. Binding of these antibodies thus depends on correct protein folding, but is independent from the stereo chemical properties of the individual aa’s. These antibodies have the further advantage of being high affinity antibodies, because the preset absorbance signal was obtained with low concentrations. Because anti-HBs immune pressure has led almost invariably to the isolation of aa 145 mutants, it is no surprise that most mAbs were very sensitive to mutations at this residue. Many antibodies were also sensitive to neighboring mutations in the second “a” loop. On the other hand, some mAbs, particularly in group 6, do not recognize mutations in a given protein region may be more reactive to replacements in another region, and thus are effective for the recognition of the latter mutants. It is an intriguing possibility that these low-affinity antibodies to wt may be responsible for the spontaneous disappearance of mutations in this genomic area. In the case of HBS.OT17, a more pronounced binding to mutant proteins was found even for both the aa 145 A and R mutants. Apart from residues 141, 144, and 145, mutations at all other residues were recognized by the panel only to a lower extend compared with wt, or were not recognized only by a limited number of mAbs. This is in accordance with the observation that these mutants are much less consistently isolated. Although the antibodies recognize native HBsAg in infected sera and Alexander cell HBsAg, all mAbs sensitive to aa145 mutations were not reactive with denatured HBsAg in Western blotting. The binding of these mAbs thus depends both on the correct folding of HBsAg and on the stereo chemical properties of the aa at residue 145 and, to a lesser degree, 141 and 144. For many mAbs, the G145R mutation compromises reactivity to a larger extent than the G145A substitution. Apparently, the polar, positively charged, and spacious side chain of R affects the conformation of the “a” loop more than A, a small aa partly resembling G. The G145R mutant causes substantial change in physicochemical terms and appears to be the quintessential immune escape mutant. Twenty of 28 antibodies (71%) did not react with this mutant. Importantly, however, we identified for the first time antibodies that bind to conformational “a” loop epitopes, yet still bind adequately to the G145R mutant, as well as to all other mutants investigated. In only one case (HBS.OT19) was reactivity also strongly reduced by the aa 133 escape mutant. The epitope of this mAb seems to be located in the first loop of the “a” domain or conformational neighboring parts of the “a” domain located at

positions 133 and 145. Antigenic changes in reactivity would be expected for Q129R, because R is larger than Q and is charged, or for T126A, because A is hydrophobic and T is polar. Our data did not show this, however, suggesting that this region is a less relevant B-cell epitope.

In conclusion, our data illustrate that G145R is the escape mutant par excellence, because none of the other mutations distributed over the “a” region caused loss of antibody binding to a comparable degree. Direct testing showing reduced HBsAg binding to monoclonal anti-HBs antibodies supports the assumption that specific conformational B-cell epitopes are altered by mutations. This effect differs, depending on the properties of the amino acids involved and on the localization within the “a” loop. Based on reactivity patterns, we classified the mAbs into groups with similar characteristics. We identified antibodies that bind to conformational epitopes of HBsAg, yet still had adequate reactivity with all escape mutants, including G145R. A group of antibodies sensitive to mutations in one region of the “a” loop had increased reactivity with mutants in another part of the “a” loop. Based on these data, selection and combination of mAbs or the development of designer antibodies with defined antibody specificity offer perspectives for the management of chronic hepatitis B complicated by the emergence of escape HBsAg mutants.

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Chapter 3: Localization of a unique hepatitis B virus epitope sheds new light on the structure of hepatitis B virus surface antigen

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Abstract

In a search for monoclonal antibodies (MAbs) that can bind hepatitis B virus surface antigen (HBsAg) with amino acid substitutions in the immune dominant “a” region (escape mutants) we investigated the epitope recognition site of the human MAb 4-7B. Pepscan analysis and experiments with alanine substitution as well as substitutions known from nature pointed to residues 178–186 in the small S protein with the amino acid sequence PFVQWFVGL (key amino acids in bold) as the minimal epitope. Single amino acid substitutions at positions 122(R/K)(d/y), 134(Y/F), 145(G/R), 148(T/A) and 160(K/R)(w/r), representing “a” region variants in recombinant HBsAg COS-I cells, did not influence binding of MAb 4-7B. Synthetic peptides (residues 175–189) including the 4-7B epitope sequence were able to evoke an anti-HBs response in rabbits. According to established polypeptide models, the 4-7B epitope region is located in the lipid layer of 20 nm HBsAg particles. The present results, however, suggest that residues 178–186 are exposed on the surface of the 20 nm particle. This may change our view of the structure of HBsAg.

Introduction

The hepatitis B virus (HBV) surface antigen (HBsAg) is the translation product of a large open reading frame that is divided into three domains. Each of these domains starts with an ATG codon functioning as a translation initiation site, thus defining three polypeptides referred to as the major protein (S protein), middle protein (M protein) and large protein (L protein). These three HBsAg polypeptides share an immune dominant and immune protective determinant located in the S region. This so-called “a” determinant is considered to be the most important target for diagnosis and immune prophylaxis. Native and recombinant forms of HBsAg exposing the “a” determinant are commonly used as the basis for hepatitis B vaccines. Such vaccines induce complete protection against HBV infection after pre-exposure HBV immunization and post-exposure maternal–infant transmission

after passive– active immunization. However, Carman et al. (1990) first described a vaccine-induced escape mutant of HBV in a child from Southern Italy who received passive–active post-exposure immunization. This escape mutant had a point mutation from guanosine to adenosine at nucleotide 587 (codon 145) in the “a” determinant. Subsequently, escape mutants induced by active or passive immunization with amino acid changes resulting in the loss of the “a” determinant were reported from other parts of the world (Carman, 1997). The first HBV marker of choice in the diagnosis of hepatitis B disease is HBsAg. Since the “a” determinant is thought to be the most immunogenic, it is to be expected that assays for HBsAg are mainly based on antibodies with “a” region preference. As a consequence even third generation assays may not detect all cases of infection with variant viruses, due to the changes in the “a” region (Carman et al., 1995, 1997; Grethe et al., 1998). To overcome this deficit we started screening a large number of monoclonal antibodies (MAbs) for their ability to detect variant HBsAg and analysed the corresponding epitopes of the most promising ones. The study described in this paper deals with the finding of a unique human HBsAg epitope located outside the “a” determinant. Surprisingly, this epitope was located within the third passage of the HBsAg polypeptide through the lipid membrane, as described in classical models (Stirk et al., 1992 ; Berting et al., 1995; Gerlich et al., 1993).

Methods

Biological material. Murine MAbs (HBs.OT16, HBs.OT17, HBs.OT24 and HBs.OT40) were developed at Organon Teknika (Boxtel, The Netherlands) according to standard methods. These MAbs are directed against native HBsAg obtained from sera of infected patients. The human MAb 4-7B-producing cell line was originally developed at the Central Laboratory of The Netherlands Blood Transfusion Services, Amsterdam. A purified preparation of MAb 4-7B was kindly provided by D. Rohm (Biotest Pharma, Dreieich, Germany)

Epitope mapping. Based on the sequence described for HBsAg subtype adr published by Fujiyama et al. (1983) 12-mer peptides were prepared covering the complete sequence by shifting one amino acid compared to each previous peptide. The reactivity of each peptide was determined by using standard pepscan procedures (Geysen et al., 1986). The minimal epitope of MAb 4-7B (shortest reactive peptide) was determined by shortening the most immune reactive 12-mer peptide at the N or C terminus and determining the reactivity of the fragments by pepscan. The importance of each individual amino acid was determined by including an alanine substitution study, in which each amino acid was replaced by alanine (A). If alanine naturally appeared in the basic sequence it was replaced by serine (S). Again the reactivity of each peptide was determined using standard pepscan technology and the most reactive 12-mer peptide was used as the basis for further study. As a control for pepscan analysis, the reactivity of the four

reactive sequences and the minimal epitope was also determined by using Ata-coupled peptides directly coated on the solid phase of a microtitre plate according to the method of Loomans et al. (1997). The synthesis of the peptides was carried out on a Perkin Elmer / Applied Biosystems 433A peptide synthesizer, using standard FastMoc 0.25 mmol procedures with UV-monitoring and feedback option. The peptides were synthesized on a TentaGel S RAM Fmoc resin via Fmoc/tBu chemistry. The linker is of a Rink-amide type, which automatically yields a C-terminally amidated peptide. During solid phase peptide synthesis the amino side chains were protected with acid-labile protecting groups : the ε-amino group of lysine with Boc, the δ-guanidino group of arginine with Pbf, the γ-carboxyl group of glutamic acid and the β-carboxyl group of aspartic acid with OtBu, the γ-amide group of glutamine and the β-amide group of asparagine with Trt, histidine and cysteine with Trt, the β-hydroxyl group of serine and threonine with tBu, and tyrosine with tBu. All reactants were dissolved in DMF. The cleavage of the Fmoc group was carried out with 20 % (v/v) piperidine in NMP during at least two consecutive cycles of 1.5 min. Coupling of the Fmoc amino acid derivatives (Fmoc-Aaa-OH, 4 eq. 1 mmol) was performed by in situ activation with HBTU, HOBt (4 eq. 1 mmol) and DIPEA. After coupling of each amino acid derivative (at least 20 min), no check for completion of the acylation reaction was carried out. The acylation was followed by a capping step with acetic anhydride in NMP. Finally, the Ata group was introduced via an active ester coupling with SAMA- OPfp. The fully protected peptides were cleaved from the resin during a 2 h reaction with 5 % (v/v) thioanisole, 3 % (v/v) ethane dithiol, 2.5% (v/v) water and 2 % (v/v) anisole in trifluoracetic acid followed by precipitation in diethyl ether. The crude peptides were washed twice in diethyl ether, dried in air, dissolved in water-acetonitrile (3:1) and lyophilized

Nomenclature as given in European Journal of Biochemistry vol. 138, pp. 9–37 (1984). (Other abbreviations: Ata, acetylthioacetyl; NMP, N-methylpyrrolidone; SAMA-OPfp, S-acetylthioglycolic acid pentafluoro-phenyl ester; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5 sulfonyl).

Reactivity of peptides with naturally appearing amino acid substitutions. Based on the sequence of the most reactive 12-mer peptide various peptides were synthesized including all known naturally appearing amino acid substitutions as described in the literature (Norder et al., 1993; Grethe et al., 1998; Loncarevic et al., 1990; Mbayed et al., 1998). The reactivity of each peptide was measured according to standard pepscan procedures.

Reactivity of recombinant HBsAg including (escape) mutants. A DNA fragment of 900 bp including the complete preS2 and S gene (subtype ayw3) was used for the construction of several point mutated DNA sequences with primer

site-directed mutagenesis. Mutant constructs in plasmid pSP70 were digested with SmaI. The HBsAg gene fragments were isolated from agarose gel with the Gene Clean II kit (BIO 101) and ligated into the blunt-ended mammalian expression vector pSG8puro at the EcoRI site under the control of the simian virus 40 promotor. The correct orientation was verified with PCR using a vector and an insert primer. We obtained single point-mutated HBsAg proteins with the following amino acids replacements : R122K, Y134F, G145R, T148A, K160R (R122K, Peterson et al., 1984; Y134F, Ashton-Rickardt & Murray, 1989; G145R, Waters et al., 1992; T148A, Ohno et al., 1993; K160R, Okamoto et al., 1989). Wild-type recombinant HBsAg was used as positive control. COS-I cells were chosen because of the high expression level, secretion into the medium and glycosylation of the translated products; $1.5*10^6$ cells were transiently transfected with 10 µg plasmid DNA in 0.2 ml PBS in a 4 mm cuvette at 300 V and 125 µF using a Biorad electroporator. Cells were cultured for 3 days in Dulbecco's modified Eagle's medium containing 10 % foetal calf serum, 2 mM glutamine, 50 µg/ml gentamycin and non-essential amino acids. Expression was controlled by Western blotting using murine MAb HBs.OT43 (Organon Teknika), directed against a linear sequence in the preS2 domain of the HBsAg product. Culture medium was harvested after 3 days and clarified by low speed centrifugation. Samples were diluted in normal human serum. Variant HBsAg was quantified in an experimental assay with preS2 MAbs recognizing linear epitopes that are not influenced by mutations in the "a" domain. Human MAb 4-7B (5 µg/ml) was coated directly on the solid phase of 96-well microtitre plates according to standard methods. Murine anti-HBs MAbs (HBs.OT16, HBs.OT17, HBs.OT24, HBs.OT40) were only available in low concentration. To achieve high solid phase concentrations of these antibodies, microtitre plates were first coated with sheep anti-mouse antibodies. Thereafter, murine anti-HBs MAbs were added. After incubation for 1 h at 37°C, extensive washing with PBS–Tween was done. Subsequently, each recombinant HBsAg was diluted to a pre-set concentration (based on the preS2 reactivity) at which the wild-type HBsAg gave an absorption signal of 1.000 at 450 nm with each individual MAb. After incubation for 1 h at 37 °C and extensive washing, sheep anti-HBs horseradish peroxidase (HRP) conjugate was added followed by incubation for 1 h at 37 °C. Tetramethylbenzidine peroxidase substrate was then added and followed by incubation for 30 min at ambient temperature. For each MAb the reaction was stopped using 1 M sulfuric acid and reactivity was measured by spectrophotometry at 450 nm. Culture medium of non-transfected COS-I cells was included as negative control.

Table 1: Mixture of peptides based on the 4-7B epitope including all known appearing variations used for immunization of rabbits. Basic sequence: LLVPFVQWFVGLSPT.

Position	Amino acid	Percentage in mixture
1	L	100
2	L	100
3	V or A	50
4	P, Q or G	33
5	F	100
6	V	100
7	Q	100
8	W, C or S	33
9	F, C or S	33
10	V, A, D or M	25
11	G or E	50
12	L	100
13	S or P	50
14	P	100
15	T or I	50

Reactivity of rabbit polyclonal anti-HBs antibodies. In order to generate antibodies against the 4-7B epitope including all possible variations (table 1), a peptide mixture of all known sequences was made. In table 1 an overview of the mixture is shown. All peptides were based on 15 amino acids and included the 4-7B epitope. Before immunization in two rabbits, peptides were coupled to keyhole limpet haemocyanin and tetanus toxoid and a pretreatment sample was taken from each rabbit. Each rabbit was immunized with 100 µg peptide (50 µg intra-muscularly and 50 µg subcutaneously, both in adjuvant). Four weeks after the first immunization a similar second immunization was carried out and 19 days later the first blood samples were taken. The reactivity of all blood samples was tested in a standard enzyme immunoassay. Plasma-derived HBsAg (both HBsAg ad and ay, 50 % each) was pretreated with pepsin to remove adhering proteins. Pepsin-treated HBsAg and a control peptide based on the 4-7B epitope (sequence 175-LLVPFVQWFVGLSPT-189) were coated on the solid phase while using swine anti-rabbit-HRP as conjugate.

Results

Epitope characterization. Pepscan analysis of the complete sequence of HBsAg subtype adr (Fujiyama et al., 1983) by 12-mer peptides revealed reactivity of MAb 4-7B with only four peptides, covering the sequence 175-LLVPFVQWFVGLSPT-189 (Fig. 1). By shortening the 12-mer peptides the minimal reactive sequence was found to be 178-PFVQWFVGL-186 (Fig. 2). Alanine substitution experiments (Fig. 3) suggest that amino acids F(179), Q(181), W(182), G(185) and L(186) are essential for reactivity of the epitope. Therefore the epitope can be determined as xxxFxQWxxGL where x can be replaced by an amino acid such as alanine. These results were confirmed in an enzyme immunoassay using Ata-coupled peptides directly coated to the solid phase of a microtitre plate.

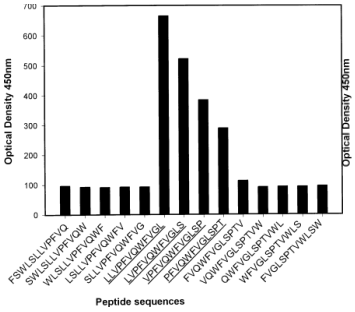

Fig. 1

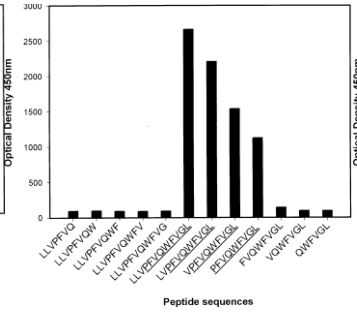

Fig. 2

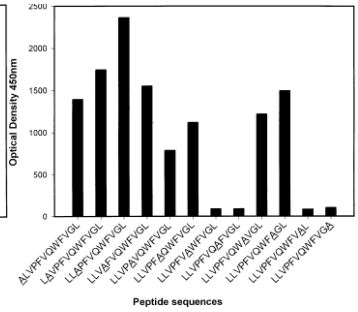

Fig. 3

Fig. 1. Pepscan analysis of MAb 4-7B on overlapping 12-mer peptides from residues 170–183.

Fig. 2. Pepscan analysis to elucidate the minimal epitope for MAb 4-7B by shortening the immune reactive 12-mer peptide (residue 175–186) at the N or C terminus.

Fig. 3. Alanine substitution in a 12-mer peptide reactive with MAb 4-7B.

Naturally occurring variation in amino acids 175–189. Table 2 illustrates the reactivity of MAb 4-7B with various peptides with naturally observed amino acid substitutions. The amino acids L(175), F(179), V(180), Q(181) and L(186) have been found to be conserved in nature (Norder et al., 1994). Alanine substitution of L(176), V(177) and V(184) had no influence on the reactivity of these peptides for MAb 4-7B. In contrast, substitution of P(178) by Q or G and V(184) by D but not by M significantly influenced their reaction. These changes in reactivity were not observed in alanine substitution experiments. It is suggested that the type of (polar) amino acid at these positions is essential. Substitution of P(178) by Q and F(183) by C are characteristic for HBsAg strain adw_4 (Norder, 1994). The decrease in reactivity upon substitution of W(182) by S or C and G(185) by E confirmed that W and G are key amino acids as observed in the alanine substitution study.

Peptide	Sequence 175-186	Reactivity A450
1	LLVPFVQWFVGL	2166
2	--A---------	2367
3	--A------A--	1753
4	-A----------	1749
5	-A-------A--	1623
6	---------M--	1577
7	---Q--------	497
8	---G--------	498
9	--------SA--	437
10	--------S---	236
11	---Q----C---	170
12	--------C---	152
13	--A------D--	132
14	---------D--	107
15	-------S----	99
16	----------E-	97
17	-------C----	93

Table 2. Reactivity of MAb 4-7B with aa 175–186 peptides including various naturally observed amino acid substitutions

MAb 4-7B reactivity with "a" region variant HBsAg. Recombinant HBsAg from COS-I cells (HBsAg/ayw$_3$) and some of the "a" region variants were used to compare the reactivity of MAb 4-7B antibodies with that of four different mouse MAbs with binding sites in the 120-160 region. Fig. 4 illustrates the impact of single amino acid substitutions in the ` a ' region on the reactivity of the mouse MAbs. For instance, the reactivity of antibody HBs.OT16 was strongly reduced if residue 145 (G) was replaced by 145 (R) or 148 (T) by 148 (A). Similar observations were made for HBs.OT40 (G145R) although the effect of the T148A substitution was less distinct. Both antibodies probably recognize a conformational epitope located inside the "a" determinant. The opposite result was observed for HBs.OT17, which showed an increased reactivity with the 145 (R) variant. The 'y' sub-determinant specificity of this antibody was clearly demonstrated by its sensitivity for a R122K substitution. HBs.OT24 could recognize all "a" region variants although its reactivity was decreased for the residue 145/148 variants. In contrast to the four mouse MAbs, MAb 4-7B reactivity was very similar for all the "a" region substitutions in these experiments. These observations are in agreement with the finding that the 4-7B epitope is located outside the "a" region. Amino acid substitutions in the 120-160 region apparently do not affect the binding of MAb 4-7B to its corresponding epitope.

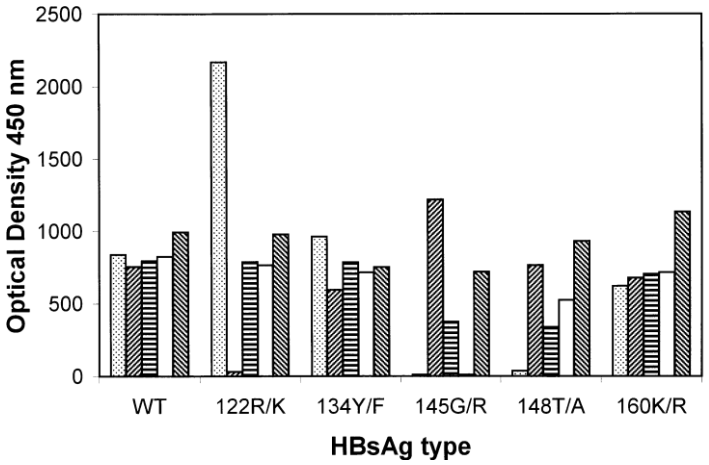

Fig. 4. Reactivity of variant HBsAg expressed in COS-I cells with MAb 4-7B (▨) and four mouse MAbs (HBs.OT16, ⁙; HBs.OT17, ▨; HBs.OT24, ▤; HBs.OT40, □) that react with "a" region epitopes. HBsAg types are designated by the residue number and substitution in the HBsAg/ayw$_3$ amino acid sequence. WT: Wild-type.

Rabbit anti-HBs induced by peptide 175–186. Two rabbits were immunized with a mixture of peptides representing all known variants in the amino acid sequence of a 15-mer based on the 4-7B epitope. As shown in Table 3 these two rabbits produced antibodies that reacted with pepsin- treated plasma-derived HBsAg directly coated on the solid phase as well as with the peptide representing the 4-7B epitope in the basic sequence only (Table 1).

Table 3. Absorbance at 450 nm of rabbit serum for antibodies reactive with plasma-derived HBsAg and a peptide (residues 175–189) including the 4-7B epitope (residues 178–186)

Serum	HBsAg		Peptide	
dilution	Rabbit 1	Rabbit 2	Rabbit 1	Rabbit 2
1:5	1848	1658	>2500	>2500
1:25	1667	1474	>2500	>2500
1:125	1313	1143	>2500	>2500
1:625	960	764	>2500	2090
1 : 3.125	534	292	>2500	662
1 : 15.625	235	83	961	218

Discussion

The cell line producing the human MAb 4-7B was first described by Stricker et al. (1985). Used for detection of HBsAg in an experimental assay MAb 4-7B could efficiently detect HBsAg while missing only one sample in 1000. Further characterization of MAb 4-7B as a suspected candidate for prophylactic use in HBsAg-positive liver transplant recipients revealed its reactivity for all strains of the Courouce panel of subtypes except for HBsAg/adw4 (Heijtink et al., 1995). In the present study peptide analysis located the MAb 4-7B corresponding epitope between amino acids 175 and 186 in the small S protein. Further refinement restricted the minimal reactive sequence (epitope) to 9 amino acids, 178-P**F**V**QW**FV**GL**-186 (key amino acids in bold). F(179), V(180), Q(181) and L(186) never change in nature. P(178) and F(183) play a crucial role in the decreased affinity of MAb 4-7B for strain HBsAg/adw_4. According to Norder (1994), a change from P(178) to Q and F(183) to C discriminates genotype F (HBsAg/adw_4) from all other genotypes. This confirms our earlier observations (Heijtink et al., 1995). W(182) and G(185) are essential amino acids as observed in substitutions experiments with alanine but also among naturally observed substitutions. The location of the MAb 4-7B epitope outside the “a” region in the classical models (Stirk et al., 1992; Berting et al., 1995; Gerlich et al., 1993) and the continuous character of the epitope suggest that MAb 4-7B binding is not disturbed by conformation changes in the “a” region. Indeed, the present study confirmed that MAb 4-7B binds easily to HBsAg variants with substitutions that have a great impact on the antigenic properties of this region (Heijtink et al., 1995). Immunization of rabbits with a mixture of the 4-7B epitope (like) peptide(s) revealed an anti-HBs response with the 4-7B epitope and pepsin-treated HBsAg(ad/ay). Studies on the prevalence of 4-7B antibodies in convalescent sera and sera from vaccinees are in progress. The proposed models for the structure of the 20 nm spherical HBsAg-containing particles carefully combined information on hydrophobicity, hydrophobic moments, flexibility, secondary structure prediction and antigenicity (Berting et al., 1995; Guerrero et al., 1988; Howard et al., 1988; Mangold et al., 1997; Stirk et al., 1992). The Stirk model for the small S protein predicts four transmembrane helices that are located between amino acid residues 8–28, 79–100, 160–184 and 189–210. Similar predictions were made by Berting et al. (1995) and Mangold et al. (1997). The model of Guerrero et al. (1988) is different from the previous models in that the peptide spans the lipid membrane six times. In this model the 4-7B epitope region would be located completely internally. According to Stirk et al. (1992) the helix C(160–184), including the 4-7B epitope region, is predicted with a low probability level. By biopanning from a filamentous phage peptide library Chen et al. (1996) obtained the amino acid sequences corresponding to four different mouse MAbs raised by plasma-derived HBsAg (Peterson et al., 1984). One of the mouse MAbs, H35, reacts with residues between 166 and 175. Another mouse MAb, H53,

corresponds to residues 187–207. Although these two MAbs recognize discontinuous epitopes with participation of amino acid residues surrounding residue 120, these examples suggest the existence of a rather extended immune reactive area between residues 160-207 including residues 176–184 as detected by human 4-7B antibodies. As mentioned above, the 4-7B epitope could be detected in all subtypes, however, with the restriction of HBsAg/adw$_4$. Furthermore, MAb 4-7B could inhibit the binding of HBsAg as well as Dane particles to solid phase anti-HBs in Ausria II (HBsAg assay, Abbott Laboratories). MAb 4-7B was also successfully used to bind the arginine residue 145 variant from cell culture (this paper) as well from human plasma (Heijtink et al., 1995). MAb 4-7B could even bind formaldehyde-treated plasma-derived vaccine HBsAg and yeast recombinant hepatitis B vaccine HBsAg (results not shown). Therefore, we conclude that the 4-7B epitope region is readily accessible in any HBsAg S polypeptide. Moreover, including the antigenic areas from MAbs H35 and H53 (Chen et al., 1996) we suggest that in 20 nm HBsAg particles the region 160–207 does not cross the lipid membrane twice but is rather projected in its full-length over the surface of these particles. In this way proline residue 188 can easily interrupt the predicted α-helix. Due to its hydrophobic character this area may be a candidate for (non-specific) binding of HBsAg particles to human hepatocytes (Leenders et al., 1990; Gerlich et al., 1993; Hertogs et al., 1993, 1994).

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Chapter 4: A new HBsAg screening assay designed for sensitive detection of HBsAg subtypes and variants.

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Abstract

The design of a new HBsAg screening assay, the Hepanostika HBsAg Ultra is based on the use of monoclonal antibodies raised against native wild type HBsAg and reactive with HBsAg in which the common “a”-determinant is modified by site-directed mutagenesis of four of the cystein moieties. The design was checked using the same cystein variants and samples from patients known infected with HBsAg variants. The results found were compared with other state of the art commercial screening assays. The design of the Hepanostika HBsAg Ultra enabled detection of all variant HBsAg positive samples in contrast to the other commercial assays. An additional 980 samples were tested to assess the specificity and sensitivity of the Hepanostika HBsAg Ultra. Screening of presumed negative serum and plasma samples resulted in a specificity of 100%. This makes the Hepanostika HBsAg Ultra the first screening assay with a design able to detect HBsAg variants with a high sensitivity and specificity.

Introduction

Hepatitis B virus (HBV) is a major public health problem with an estimated 350 million persistent carriers [1]. In the course of infection with HBV an array of antigens and antibodies can be detected in the blood. The first marker that appears after infection with HBV is the hepatitis B surface antigen (HBsAg), which can be detected in serum 4-10 weeks after infection and is rapidly followed by antibodies against the Hepatitis B core antigen (anti-HBc) [2]. Depending on the course of infection, HBsAg titers decline and protective antibodies against HBsAg (anti-HBs) become detectable. In 5-10% of the infections, the host does not clear HBV and HBsAg remains detectable, often for life [2].

HBsAg is a complex macromolecule, consisting of glycoproteins embedded in lipids derived from the host endoplasmic reticulum [3] and its major antigenic determinant, called the common “a”-determinant, is located between amino acids 100-160. The common “a”-determinant contains 8 cysteins believed to form inter- and intra-chain disulfide bridges [4, 5], dividing the “a”-determinant in distinct immunological regions [6]. Mutagenesis of these cysteins has shown that the cysteins are very important for the immunological recognition of HBsAg [7, 8].

To increase the safety of the blood supply, blood is screened for the presence of HBsAg, sometimes in combination with screening for the presence of anti-HBc antibodies to exclude past HBV infections [9- 13].

Despite the high performance of the HBsAg screening assays, transfusion-associated HBV infection is still reported [13, 14]. These false-negative results might have different causes: In chronic HBV carriers, the HBsAg level may be below the detection limit either or not combined with mutations which renders the HBsAg screening assay negative [15-17]. Another possibility is the presence of variants that are not recognized by the antibodies used in the screening assay [18-24] or immune complexes masking HBsAg epitopes [13, 25].

The Hepanostika HBsAg Ultra is designed to ensure highly sensitive broad detection of HBsAg in serum and plasma samples. Since it is known that most mutations occur in the common “a”-determinant, we designed a HBsAg screening assay with a mixture of capture monoclonal antibodies, including a unique human monoclonal antibody reactive to an epitope located outside the common “a”-determinant [26]. In previous structural models the binding region of the human monoclonal antibody 4-7B (amino acid 178-186) is presumed to be located inside the HBsAg particle [27, 28], but we and others have shown that this region is exposed on the surface of this particle [26, 29-31].

In this study we validate our assay design by testing cystein variants located in the common “a”-determinant and compare the performance with well-established serological screening assays. The sensitivity of the Hepanostika HBsAg Ultra is determined using HBsAg dilution series and finally the broad detection of the Hepanostika HBsAg Ultra is compared with other serological screening assays currently in the market using HBsAg from naturally occurring variants. The specificity was assessed by testing serum and plasma from healthy blood donors.

Material and methods

Monoclonal antibodies

All murine monoclonal anti-HBs antibodies (HBs.OT39, HBs.OT40, HBs.OT48) were produced by bioMérieux according to standard methods. Pepsin treated or native wild type HBsAg of subtype ad/ay prepared from pooled plasma was used as antigen. The human monoclonal antibody 4-7B-producing cell line was originally developed at the Central Laboratory of The Netherlands Blood Transfusion Services, Amsterdam [32].

Generation of cystein substitution variants

The cystein substitution variants C124R, C137Y, C138W and C147S in the small S-protein were prepared by site-directed mutagenesis and expressed in HepG2 cells. In short, a 900bp fragment coding for the middle S-protein of subtype ayw3 (genotype D) was used for the construction of the sequences. The mutated sequences were cloned in a mammalian expression vector pSG5 (Stratagene). Expression of HBsAg was achieved by transient transfection of 5µg DNA (endofree Maxiprep, Qiagen) using Fugene6 (Roche) on 10^5 HepG2 cells. After three days, cell culture supernatant was collected and clarified by centrifugation at 1000xg. The recombinant HBsAg variants were diluted to 0.25 U/ml in NHS prior use, as determined in an experimental test system with an anti-PreS2 monoclonal antibody coated on the solid phase.

Hepanostika HBsAg Ultra

Antibodies in the Hepanostika HBsAg Ultra were selected based on their different binding properties with cystein variants and native HBsAg. For this purpose we selected four cystein variants representing different potential binding areas within the common “a”-determinant (figure 1). The C124R variant should affect monoclonal antibodies reactive within the micro conformation including amino acids (aa) 121-124. The C137Y variant should affect monoclonal antibodies dependent on the conformation of the region between aa 107-137. The C138W and C147S variants should affect monoclonal antibodies dependent on the conformation of the region between aa 138 and 149, which is stabilized by two cystein bridges.

Microtiter plates are coated with murine and human monoclonal antibodies (separate or mixtures) for detection of HBsAg positive samples. Bound HBsAg is detected with ovine anti-HBs antibodies coupled to horseradish peroxidase (HRP). 25µl specimen diluent is added to the well followed by 100µl sample. After 1-hour incubation at 37°C, 50µl conjugate is added and incubated at 37°C for 1-hour. Unbound conjugate is removed by extensive washing with PBS-Tween and HBsAg/anti-HBs HRP is detected by addition of TMB/PO substrate, which gives a blue color in the presence of HRP. After 30 minutes substrate incubation, the reaction is stopped with 1M H_2SO_4 and positive wells appear in yellow. The absorbance is read at two wavelengths, A450nm and A690nm as reference, in an automated plate reader from bioMérieux. Samples are considered positive when the signal is greater than the average signal of the negative control plus a fixed value of 0.040 AU.

Preliminary assessment of the performance of the Hepanostika HBsAg Ultra

Clinical specificity

The preliminary assay performance of the Hepanostika HBsAg Ultra was assessed with 482 EDTA plasma and 479 serum samples from a low endemic population (Belgium). The samples were prescreened for HBsAg and found negative with the Abbott Prism HBsAg test.

Analytical sensitivity

The sensitivity of the Hepanostika HBsAg Ultra was determined with the AFSSaPS (Agence Française de Sécurité Sanitaire des Produits de Santé (the former AdM (Agence du Médicament)) panel, which consists of nine samples with decreasing concentrations of HBsAg ranging from 2.22 to 0.06ng/mL.

Subtype reactivity

The subtyped HBsAg samples in the AFSSaPS panel were tested to demonstrate broad subtype reactivity.

Variant detection

Clinical samples collected from patients infected with known HBsAg variants were tested to challenge the broad reactivity. The samples were diluted in normal human serum to an S/Co ratio of 3 or higher in the Hepanostika HBsAg Ultra. The commercial assays were performed according to the manufacturers instructions for use and the study was performed with manual sample addition and using the following equipment; Washer 430 bioMérieux, Incubator 50X bioMérieux, Reader 530 bioMérieux.

Results

Design of the Hepanostika HBsAg Ultra

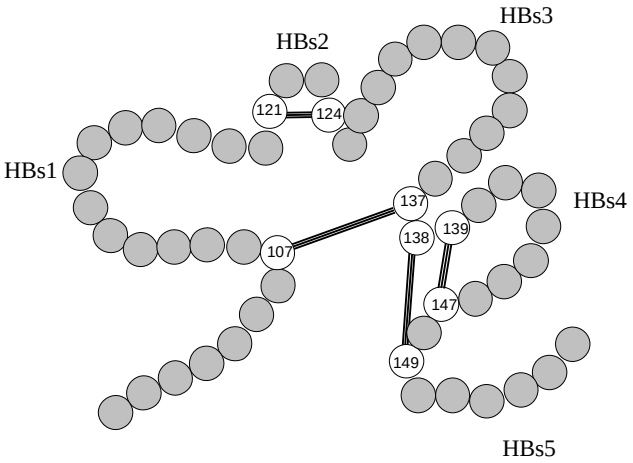

Figure 1: Proposed model according to Wallace et al. for the common “a”-determinant [6]. The numbered positions of the 8 cysteins and proposed disulfide bridges are indicated.

The Hepanostika HBsAg Ultra is designed for high sensitive broad detection of HBsAg. Hereto a panel of monoclonal antibodies was selected based on their reactivity with cystein variants of the major antigenic region of HBsAg, the common “a”-determinant (figure 1). Table 1 illustrates the difference in reactivity with this cystein substitution panel. The monoclonal HBs.OT39 is clearly reactive with wild type HBsAg, but diminished or no reactivity was observed with the cystein variants tested. This in contrast to the other antibodies tested. The monoclonal antibody HBs.OT40 is reactive with all variants except the C138W variant. The monoclonal antibody HBs.OT48 was reactive with all except the C124R variant. The human monoclonal 4-7B, as its binding site is located outside the common “a”-determinant [26], is able to recognize all HBsAg samples tested. To ensure detection of all HBsAg variants with mutations in different parts of the common “a”-determinant, the monoclonal antibodies HBs.OT40, HBs.OT48 and 4-7B were selected reactive with different cystein variants. This should result in a HBsAg screening assay that can withstand mutations in different parts of the small S-protein. To confirm this, the same recombinant HBsAg samples were tested and compared to three commercial assays (table 1).

Table 1: Reactivity of monoclonal antibodies with recombinant HBsAg variants. The Hepanostika HBsAg Ultra (HBs.OT40, HBs.OT48 and 4-7B) was tested with the same variants and compared to three commercial assays (A, B, C). The values below the cut-off are marked gray. Reactivity (signals over cut-off or S/CO) lower than the reactivity found with other monoclonal antibodies or other screening assays are marked bold.

	monoclonal antibody				S/Co			
Sample	HBs.OT39	HBs.OT40	HBs.OT48	4-7B	Ultra	A	B	C
Wild type	1157	1312	**89**	641	8,64	11,20	7,32	25,55
C124R	**239**	2159	neg	2180	36,90	31,12	neg	neg
C137Y	neg	1329	**225**	1495	29,22	**8,96**	neg	30,95
C138W	neg	neg	742	1054	15,51	**1,41**	neg	15,62
C147S	neg	1477	1291	1107	19,94	6,85	neg	21,03

The Hepanostika HBsAg Ultra and commercial assay A detected all cystein variants, however the reactivity of two HBsAg variants, C137Y and C138W, were clearly low reactive in commercial assay A (compare wild type reactivity with reactivity C137Y and C138W). The commercial assay B was not able to detect any of the cystein variants. The commercial assay C was able to detect all variants except the C124R variant, this sample was completely unreactive. HBsAg variant samples from patients infected with HBV were tested in the Hepanostika HBsAg Ultra and compared to the reactivity found in commercial assay A, B and C (table 2). We found that all samples were positive in both the Hepanostika HBsAg Ultra and commercial assays A and C, while none of the samples were positive in the commercial assay B. This demonstrates that the design of the Hepanostika HBsAg Ultra is suited to detect all variant HBsAg positive samples.

Table 2: HBsAg variants collected from patients known infected with HBV. The samples were tested in the Hepanostika HBsAg Ultra and three commercial assays (A, B, C) according to the manufacturer's instructions for use. The samples were diluted in normal human serum to an S/Co ratio of 3 or higher in the Hepanostika HBsAg Ultra. nt = not tested. Negative values are indicated in gray.

	S/Co				
Sample	**Ultra**	**A**	**B**	**C**	**Dilution**
HBsAg/ayw, G145R	18,28	22,00	Neg	29,79	10000
HBsAg/adw2, G145R/L173P	27,09	36,03	Neg	46,77	100000
HBsAg/adr, D144E/G145R	3,02	4,99	Neg	nt	1000
HBsAg/ayw3, D144E/G145R	5,92	3,38	Neg	5,75	100000
HBsAg/Ayw3, S143L: HBV6278-04	3,09	5,39	Neg	nt	undiluted

Preliminary assessment of the performance of the Hepanostika HBsAg Ultra

The Hepanostika HBsAg Ultra was tested with 482 EDTA plasma and 479 serum samples that were found HBsAg negative in the Abbott Prism. None of the tested samples were found above a sample over cut-off ratio (S/Co) of 0.6. The distribution of the serum and plasma samples (figure 2) was found highly comparable, the average signal over cut-off (S/Co) of the plasma population, 0.201 S/Co, was slightly below the average S/Co of the serum population, 0.213 S/Co. The standard deviation of the population was 0.046 S/Co for the plasma population and 0.053 S/Co for the serum population, confirming the high specificity of the Hepanostika HBsAg Ultra.

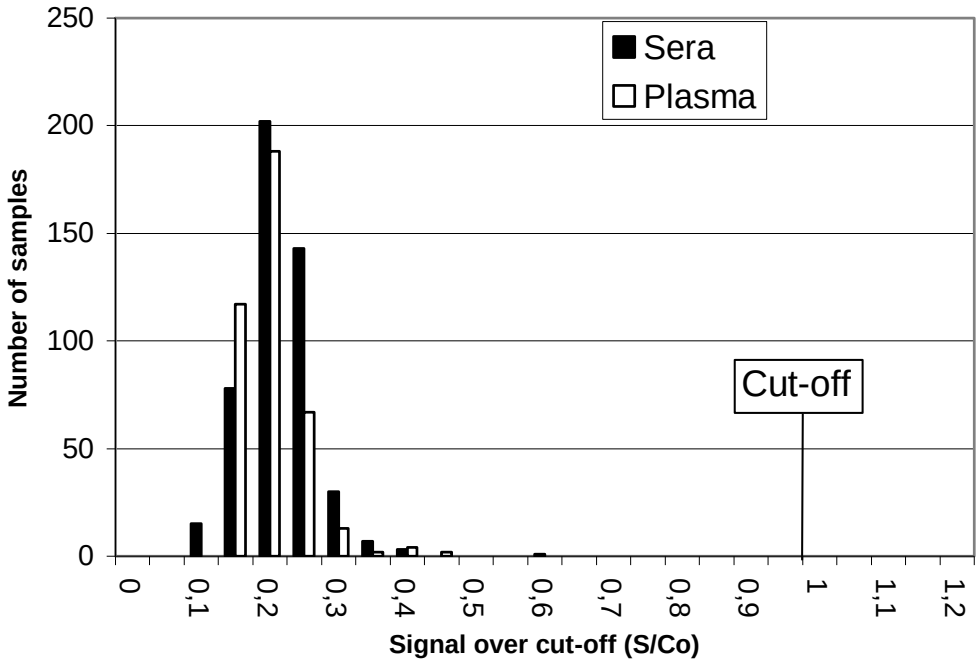

Figure 2: Frequency distribution of 482 EDTA plasma and 479 sera in the Hepanostika HBsAg Ultra. The frequencies of serum samples are indicated with black bars and the plasma samples with white bars.

The sensitivity of the Hepanostika HBsAg Ultra was tested using the AFSSaPS panel. We found that the Hepanostika HBsAg Ultra was able to detect panel member 109 (0.12ng/ml), while panel member 110 (0.06ng/ml) was found below the cut-off. In addition, all the HBsAg subtypes of the AFSSaPS panel were detected.

Discussion

The Hepanostika HBsAg Ultra was designed using cystein variants affecting different areas in the common “a”-determinant. Earlier studies showed that replacement of one or more cyteins affected the secretion and folding of HBsAg [4], but also showed that the binding of antibodies directed against HBsAg was affected [7, 33, 34]. In addition, it was shown that HBsAg cystein variants were not detected in diagnostic assays [4]. Based on published models of the common “a”-determinant [6] we selected four cystein variants that should affect the confirmation of different regions in the common “a” determinant, also known as HBs1 – HBs5 [6]. We found that the selected monoclonal antibodies for the Hepanostika HBsAg Ultra each displayed a different reaction pattern with these cystein variants, which indicates that the antibodies have different binding regions within the common “a”-determinant. Monoclonal HBs.OT40 was not reactive with the cystein variant C138W and this suggests that the monoclonal binds in the HBs4 region, amino acid 138-149. The monoclonal HBs.OT48 was not reactive with the C124R cystein variant suggesting that this monoclonal binds to the HBs2 region, amino acid 121-124. Others reported that antibodies reactive in this region were also affected by the 121 and 124 cystein substitutions [35, 36], while other cystein substitutions did not affect the binding of the similar antibodies. The region 121-124 is highly conserved in almost all published HBsAg sequences [36, 37] and therefore the use of antibodies reactive to this region ensures broad detection of HBsAg. Finally, the monoclonal 4-7B [26] was not affected by any of the cystein variants and confirms that the monoclonal binds to a region outside the common “a”-determinant. Based on these results we can conclude that the combination of three antibodies ensures detection of HBsAg, since it is unlikely that a mutant will arise with combined mutations in the three binding areas of these antibodies and still result in a viable replicating virus.

The Hepanostika HBsAg Ultra was compared with other HBsAg screening assays on the capability of recognizing the four selected cystein variants. Commercial assay A is able to detect all cystein variants, but the selected combination is dependent on the correct conformation of the common “a”-determinant. The ability to detect all clinical variant HBsAg samples with mutations in the HBs4 region is confirmed by the detection of the cystein variants of this same region. It was striking to see that commercial assay B was not able to detect even one cystein variant. The use of conformation dependent antibodies resulted in an impaired mutant detection. This observation is confirmed with HBsAg variants with mutations in the HBs4 region. The commercial assay C relies on the use of antibodies in the highly conserved HBs2 region, which is confirmed by detection of all clinical samples from known HBsAg variants with mutations in the HBs4 region. However, it remains possible that HBsAg variants are missed in this assay, especially the ones with mutations in this HBs2 region. Several reports have been published

describing variants with mutations in the HBs2 region, although the frequency at which these variants occur is hard to establish from this data [19, 23, 38].

The preliminary specificity of the Hepanostika HBsAg Ultra was established with 482 EDTA plasma and 479 sera, proving excellent specificity. The clinical sensitivity was challenged with the AFSSaPS panel, and showed a comparable or better performance when compared to other diagnostic assays [39].

In conclusion: The Hepanostika HBsAg Ultra is the first HBsAg screening assay with a proven design able to detect variants having extreme changes in the common “a”-determinant. We showed and it is published that other diagnostics assays fail to detect certain HBsAg variants [18, 20-24]. The need for a screening assay able to detect all HBsAg variant was recently reported in a consensus meeting [13] and with the introduction of the new Hepanostika HBsAg Ultra we can answer this need.

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Chapter 5: Evaluation of the Hepanostika HBsAg Ultra and confirmatory assay: a new hepatitis B surface antigen (HBsAg) screening assay combination designed for sensitive and specific detection of HBsAg subtypes and variants

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Abstract

The Hepanostika HBsAg Ultra screening assay was assessed with 40 commercial available seroconversion panels and 445 HBsAg positive sera in comparison with two other CE Marked HBsAg screening assays. The ability to detect HBsAg variants was investigated with 11 recombinant expressed HBsAg variants and 9 natural HBsAg variants compared to three other CE-Marked HBsAg screening assays. The limit of detection was determined with the AFSSaPS and WHO HBsAg standards and finally the specificity of the Hepanostika HBsAg Ultra screening assay was evaluated with 4132 sera and 20727 EDTA plasmas from the Belgian, Danish and Dutch donor population. Repeat reactive samples found in the Hepanostika HBsAg Ultra assay were tested in the Hepanostika HBsAg Ultra confirmatory assay to verify its ability to correctly confirm the presence of HBsAg and not to confirm non-specific reactive samples. Comparison of the Hepanostika HBsAg Ultra screening assay with the ORTHO Antibody to HBsAg ELISA Test System 3 and Murex HBsAg Version 3 revealed that the Hepanostika HBsAg Ultra screening assay was more sensitive than the Ortho assay and had a similar sensitivity compared to the Murex assay. The limit of detection of the Hepanostika HBsAg Ultra assay was found to be 0.04 ± 0.002 IU WHO HBsAg /ml and 0.08 ± 0.004 AFSSaPS HBsAg ng/ml, confirming the clinical sensitivity. All HBsAg variants were detected with the Hepanostika HBsAg Ultra assay while the other HBsAg screening assays failed to detect one or more of the tested HBsAg variants. The specificity of the Hepanostika HBsAg Ultra assay was found 99.95% after repeat testing. The verification of the Hepanostika HBsAg Ultra confirmatory assay with HBsAg positive and repeat reactive HBsAg negative samples revealed that all samples were correctly confirmed. In conclusion, the Hepanostika HBsAg Ultra assay proved to be a highly sensitive HBsAg screening assay able to detect all HBsAg positive samples including HBsAg variants, with a high specificity.

Introduction

Hepatitis B virus infection (HBV) is a major public health problem with an estimated 350 million persistent carriers (1). In the course of an HBV infection an array of antigens and antibodies can be detected in the blood. The first marker that appears after infection with HBV is the hepatitis B surface antigen (HBsAg), which can be detected in serum 4 to 10 weeks after infection and is rapidly followed by antibodies against the Hepatitis B core antigen (anti-HBc) (2). Depending on the course of the infection, HBsAg levels decline and protective antibodies against HBsAg (anti-HBs) become detectable. In 5 to 10% of the infections, the host does not clear HBV and HBsAg remains detectable, often for life (2). To increase the safety of the blood supply, blood and related products are screened for the presence of HBsAg, sometimes in combination with screening for the presence of anti-HBc antibodies to exclude past HBV or occult HBV infections (3–7).

Despite the high performance of the HBsAg screening assays, transfusion-associated HBV infection is currently still being reported (7,8). These false-negative results might have different causes: in chronic HBV carriers, the HBsAg level may be below the detection limit either or not combined with mutations which renders the HBsAg screening assay result negative (3,9,10). Another possibility is the presence of variants that are not recognized by the antibodies used in the screening assay (11–17) or immune complexes masking HBsAg epitopes (7,18).

In a previous study we presented a new HBsAg screening assay (Hepanostika HBsAg Ultra) designed to detect HBsAg and HBsAg variants, including those with cysteine residue substitutions in the common “a”-determinant (19). In this preliminary study the analytical sensitivity and specificity of the Hepanostika HBsAg Ultra assay was assessed with limited numbers of sera and plasma samples. In the present study we present the result of a study on clinical sensitivity, analytical sensitivity and specificity of the Hepanostika HBsAg Ultra compared to other well-established serological assays. The specificity of the Hepanostika HBsAg Ultra was assessed with large (>24 000) numbers of sera and plasma samples from healthy donors obtained from three different blood banks. In addition, the performance of the Hepanostika HBsAg Ultra confirmatory assay was verified with repeat reactive samples in order to establish its specificity and suitability to confirm HBsAg reactive samples found with the Hepanostika HBsAg Ultra screening assay.

Material and Methods

Hepanostika HBsAg Ultra

Microtiter plates are coated with murine and human monoclonal antibodies for detection of HBsAg. Bound HBsAg is detected with ovine anti-HBs antibodies conjugated to horseradish peroxidase (HRP). Twenty-five µl specimen diluent is added to the well followed by 100µl serum or plasma sample. Sample addition is monitored at 405 nm with 690 nm as reference filter and is confirmed when an absorbance of greater than 0.1 AU is recorded. After 1-hour incubation at 37°C, 50 µl conjugate is added and incubated at 37°C for 1-hour. Conjugate addition is measured at 620 nm with 690 nm as reference filter and is confirmed when an absorbance of greater than 0.5 AU is recorded. Unbound conjugate is removed by extensive washing with PBS-Tween and the HBsAg/anti-HBs-HRP complex is detected by addition of TMB/PO substrate, which gives a blue color in the presence of HRP. After 30 minutes substrate incubation, the reaction is stopped with 1 M H_2SO_4 and HBsAg positive wells appear in yellow. The absorbance is read at two wavelengths, A450nm and A690nm as a reference, in an automated bioMérieux plate reader. Samples are considered positive when the signal is greater than the average values of the negative controls plus a fixed value of 0.040 AU (cut-off).

Hepanostika HBsAg Confirmatory

Repeat reactive samples in the Hepanostika HBsAg Ultra can be confirmed using the Hepanostika HBsAg Confirmatory assay. To this end 100 µl of the presumed HBsAg positive sample is transferred to an empty uncoated well with 25µl control reagent and to an empty well with 25 µl neutralizing reagent. After 16 to 24hours incubation at room temperature, both samples are tested as regular samples in the Hepanostika HBsAg Ultra screening assay. Samples are considered confirmed positive when the signal of the sample with control reagent is greater than the average signal of the negative control plus a fixed value of 0.025 AU (cut-off) and when the signal from the neutralized sample is at least 50% lower than the signal with the control sample. If both the signal of the control and neutralized sample exceeds 2.000 AU the sample is diluted in 0.15 M NaCl and retested.

Assay performance

Diagnostic sensitivity

The diagnostic sensitivity of the Hepanostika HBsAg Ultra was determined using in total 40 seroconversion panels from SeraCare (SeraCare Life Sciences, Inc.) and ZeptoMetrix (ZeptoMetrix Corp.). For comparison, the panels were also tested in the ORTHO Antibody to HBsAg ELISA Test System 3 and the Murex HBsAg Version 3 (Abbott-Murex).

HBsAg positive samples

HBsAg positive sera (n=445) from patients during various stages of Hepatitis B virus infection (SeraCare Life Sciences, Inc.) were tested in the Hepanostika HBsAg Ultra and compared to the ORTHO Antibody to HBsAg ELISA Test System 3. The high positive HBsAg samples were not tested in the ABBOTT Murex HBsAg Version 3 due to the limited volume of most of the samples. High dose hook effect was investigated with 7 HBsAg positive samples containing at least 10 000 ng/ml HBsAg calibrated against the AFSSaPS (Agence Française de Sécurité Sanitaire des Produits de Santé) HBsAg standard (mixture of HBsAg/adw2 and HBsAg/ayw3). The high dose hook effect may be observed in theory when excess of antigen is bound in complex with conjugate so that this complex is not captured on the solid phase.

HBsAg standards

The limit of detection (LOD) was calculated using the second international standard (WHO) for HBsAg (subtype adw2, genotype A) NIBSC code 00/588, which contains 33 IU HBsAg / vial and the AFSSaPS HBsAg standard. The freeze-dried international HBsAg standard was reconstituted in water and used to prepare the prescribed 1/4, 1/16, 1/64 and 1/256 dilutions in normal human serum supplied with the AFSSaPS HBsAg standard. Besides the prescribed dilutions, additional dilutions were prepared in the cut-off range of the Hepanostika HBsAg Ultra assay in order to accurately determine the LOD of the WHO standard.

The AFSSaPS standard was tested as prescribed and for a more accurate calculation of the LOD we diluted the 5ng/ml AFSSaPS HBsAg standard in the normal human serum supplied by the AFSSaPS in the complete measuring range of the Hepanostika HBsAg Ultra assay and around the assay cut-off. The data found with two independent production lots were statistically analyzed and used to calculate the LOD and the conversion factor between the AFSSaPS standard and the international HBsAg standard.

Specificity

The specificity of the Hepanostika HBsAg Ultra assay was assessed with 4132 sera and 20727 EDTA plasmas from the Belgian, Danish and Dutch donor population in four independent studies and using five production lots. The samples from the Belgian and Dutch donor population were prescreened using the ABBOTT PRISM and only the negative donations were used in our study. The specificity study was assessed with manual equipment or using the DAVINCI® instrument, a full automated high-volume micro Elisa immune analyzer. A total of 340 samples from hospitalized patients from different categories were tested to check for potentially interfering factors. In addition, 52 matched serum and plasma samples (EDTA, Citrate and Heparin) were tested to demonstrate compatibility of different sample types in the Hepanostika HBsAg Ultra assay. All reactive samples were

retested in the Hepanostika HBsAg Ultra assay and the ORTHO Antibody to HBsAg Elisa Test System 3.

HBsAg variants and clinical samples know positive for HBV

The broad reactivity of the Hepanostika HBsAg Ultra assay was challenged using a panel of recombinant and clinical samples. Recombinant HBsAg was expressed in HepG2 as described previously (19). All samples were diluted in normal human serum to an S/Co ratio of 3 or higher in the Hepanostika HBsAg Ultra assay, in order to save sample as well as to be able to critically judge the ability to detect HBsAg variants as compared to other state-of-the-art screening assays: ORTHO Antibody to HBsAg ELISA Test System 3, ABBOTT Murex HBsAg Version 3 and ABBOTT PRISM HBsAg. The Ortho and the Murex HBsAg assays were used according to the manufacturer's instructions for use with manual sample addition and the following equipment; Washer 430 bioMérieux, Incubator 50X bioMérieux, Reader 530 bioMérieux.

Confirmation of repeat reactive samples

Repeat reactive samples (N=90) from the Hepanostika HBsAg Ultra screening assay were tested in the Hepanostika HBsAg Ultra confirmatory assay as part of the verification and validation of the Hepanostika HBsAg Ultra confirmatory assay. The repeat reactive samples were confirmed negative for HBsAg after testing in the ABBOTT Murex HBsAg Version 3 and the Genetic Systems® HBsAg EIA 3.0. Critical HBsAg variants, HBsAg subtypes and high positive samples were tested to proof ability to neutralize HBsAg positive samples found throughout the world.

Results

Diagnostic sensitivity

The diagnostic sensitivity of the Hepanostika HBsAg Ultra was assessed with 40 seroconversion panels. The Hepanostika HBsAg Ultra detected most of the samples within the seroconversion panels earlier, compared to the ORTHO Antibody to HBsAg ELISA Test System 3 (table 1). In 68 % (27/40) of the seroconversion panels one or more samples were detected earlier in the Hepanostika HBsAg Ultra. This in contrast to the Abbott/Murex HBsAg version 3 were we found two seroconversion panels that were detected differently by the Hepanostika HBsAg Ultra and the Abbott/Murex HBsAg version 3 assay. In one seroconversion panel the Hepanostika HBsAg Ultra detected one sample more than the Abbott/Murex HBsAg version 3 and in the other seroconversion panel the Abbott/Murex HBsAg version 3 assay detected one sample more than the Hepanostika HBsAg Ultra (table 1). In all cases these samples were either just above the cut-off in the Hepanostika HBsAg Ultra assay or just above the cut-off in the Abbott/Murex HBsAg version 3, which indicates that the diagnostic sensitivity of both assays is comparable (Hepanostika Vs Murex: 1.05 vs 0.94 and 0.94-0.89 vs 1.08-1.05).

Table 1: Diagnostic sensitivity of HBsAg screening assays with seroconversion panels. The different panels are indicated in the first column next to the number of samples in the panel. On each row the first sample found positive is presented except for panel phm935b, here the last reactive sample is indicated. All gray cells indicate when the Hepanostika HBsAg Ultra assay is the first assay to detect HBsAg. All bold and underlined cells indicate seroconversion panels where the Hepanostika HBsAg Ultra assay is later in detecting HBsAg.

			Hepanostika HBsAg Ultra	**Ortho Antibody to HBsAg**	**Murex HBsAg version 3**
Panel		Number of samples	first/last* positive sample		
SeraCare	phm902	14	8	9	nt
	phm903	6	4	5	nt
	phm904	3	2	3	nt
	phm906	5	2	2	nt
	phm909	7	4	5	nt
	phm910	6	3	3	nt
	phm911	25	20	21	nt
	phm912	9	8	8	nt
	phm915(m)	13	7	10	**4**
	phm916	11	9	10	9
	phm917	3	3	3	3
	phm918	3	2	3	2
	phm919	9	6	6	6
	phm920	6	3	3	3
	phm921	6	1	1	1
	phm922	12	6	7	6
	phm923	4	3	3	3
	phm925	5	3	4	3
	phm927	6	2	3	2
	phm928	7	4	5	5
	phm929	9	5	6	5
	phm930	5	2	2	2
	phm931	8	5	6	5
	phm932	16	10	10	10
	phm933	6	3	4	3
	phm934	6	1	2	1
	phm935a	20	7	8	7
	phm935b*	12	32	25	32
ZeptoMetrix	don 6271	5	3	4	nt
	don 6272	26	23	25	nt
	don 6284	19	13	14	13
	don 6285	16	11	11	11
	don 6286	9	5	6	5
	don 6287	11	10	10	10
	don 6288	9	5	6	5
	don 6289	10	7	8	7
	don 6290	12	7	8	7
	don 6291	8	6	6	6
	don 6292	12	7	8	7
	don 6293	7	4	5	4

* Last reactive sample indicated.

HBsAg positive samples

High positive HBsAg samples (n=445) were tested in both the Hepanostika HBsAg Ultra and the ORTHO Antibody to HBsAg ELISA Test System 3. Initially, 444 HBsAg positive samples were detected in both assays and one sample was below the cut-off in the ORTHO Antibody to HBsAg ELISA Test System 3. Retesting of this negative sample in the ORTHO Antibody to HBsAg ELISA Test System 3 confirmed the initial data, i.e., the sample remained positive in the Hepanostika

HBsAg Ultra assay and negative in the ORTHO Antibody to HBsAg ELISA Test System 3. In the high range (>10 000ng/ml HBsAg), no high dose hook effect was observed in both the Hepanostika HBsAg Ultra assay and in the ORTHO Antibody to HBsAg ELISA Test System 3. Analysis of 83 high positive samples reactive in the measurement range of both screening assays (i.e. below an absorbance of 3000mAU) showed that on average the Hepanostika HBsAg Ultra assay is about two times more sensitive than the ORTHO Antibody to HBsAg ELISA Test System 3 (figure 1).

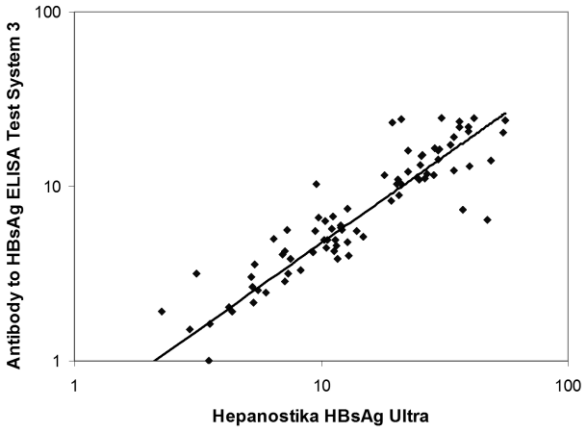

Figure 1: The reactivity of high HBsAg positive samples in the Hepanostika HBsAg Ultra assay vs the Ortho antibody to HBsAg ELISA Test System 3. The x-axis represents the signal over cut off (S/Co) found with the Hepanostika HBsAg Ultra assay and the y-axis the S/Co found with the Ortho antibody to HBsAg ELISA Test System 3. The line represents a linear regression on the found data with the formula y=0.4716x.

HBsAg standards

Two independent production lots of the Hepanostika HBsAg Ultra assay were tested with dilution series of the WHO and the AFSSaPS HBsAg standard. Using the obtained data, a conversion factor between the two standard preparations was calculated by linear regression of the ^{10}log transformed response data upon the ^{10}log transformed input concentrations (figure 2). The conversion factor was calculated to be 0.50 IU WHO/ng AFSSaPS with a 95 % confidence interval ranging from 0.49 to 0.51 (IU/ng). Statistical analysis (F-tests) for any of the definable interaction effects in the linear models (ANCOVAs) revealed no significant plate effect, assay lot effect or difference in WHO/AFSSaPS panel reactivity (curve linearity) ($p<0.092$). The calculated conversion factor was found in range with the reported conversion factor of 0.518 (IU/ng) (20). The limit of detection was calculated using linear regression and an assay cut-off of average negative controls + 0.040 AU. We found a sensitivity of 0.04 ± 0.002 IU WHO /ml

or 0.08 ± 0.004 AFSSaPS ng/ml. The linearity of the AFSSaPS dilution series matched well with the pre-diluted AFSSaPS series, i.e. all positive samples from the pre-diluted AFSSaPS series were found within the 95 % confidence limits of the gravimetrically prepared dilution series (figure 3). Below the cut-off (dashed line) we found a lack of correlation with the pre-diluted AFSSaPS series and the calculated line, but with the intervals given by AFSSaPS (depicted as horizontal bars in figure 3) it seems reasonable that the pre-diluted AFSSaPS series can be part of gravimetrically prepared dilution series (figure 3).

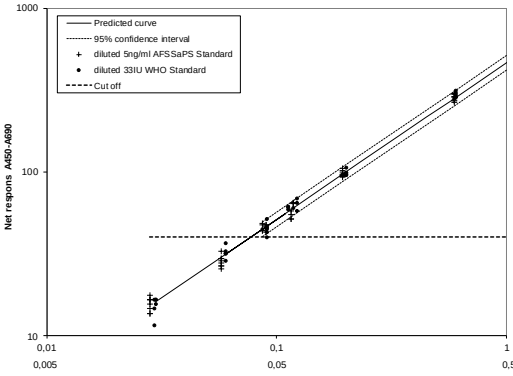

Fig 2

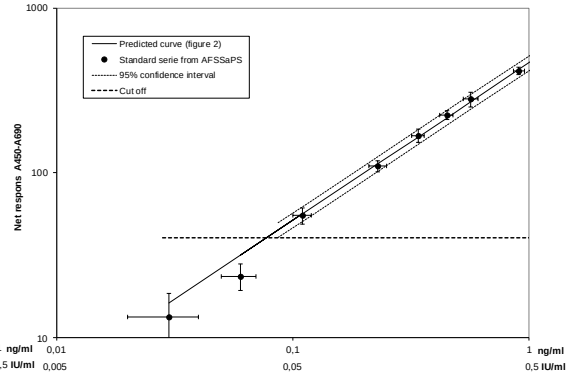

fig 3

Figure 2: The reactivity of diluted 5ng HBsAg /ml (AFSSaPS) and 33 IU HBsAg (WHO) standard. The data found with the diluted WHO standard (+) and AFSSaPS standard (●) was used to calculate the linear line. The corresponding 95% confidence interval and cut off are plotted as dashed lines. **Figure 3**: The reactivity of the pre-diluted AFSSaPS series compared to the calculated line (figure 2). The data found with the pre-diluted AFSSaPS series (●) and the corresponding standard errors are given as horizontal and vertical bars. The 95% confidence interval of the calculated line and cut off are plotted as dashed lines.

Specificity

The specificity of the Hepanostika HBsAg Ultra assay was assessed using prescreened HBsAg negative samples. Data from routine use of the Hepanostika HBsAg Ultra assay on the DAVINCI instrument was included for a more precise estimate of specificity. Testing of two independent batches Hepanostika HBsAg Ultra assay on prescreened HBsAg negative samples (ABBOTT PRISM) from Belgian and Dutch donors, revealed a specificity of 99.95%. The distribution of the negative samples in both production lots was highly similar; i.e. no difference was noted between the plasma and serum samples tested in both lots (data not shown). Routine use of the Hepanostika HBsAg Ultra assay on the DAVINCI instrument confirmed the initial findings found with the plasma samples (Figure 4, Danish). The specificity was found 99.95% after testing in total 20727 plasma samples using three production lots. Compatibility of Hepanostika HBsAg Ultra assay with serum and types of plasma (EDTA, Citrate and Heparin) was assessed with serum and

plasmas collected from the same donor (N=52). All samples were found negative and no systematic difference in reactivity between the different sample types could be detected. Spiking of the same samples with a fixed amount of HBsAg did not reveal any interference of the anticoagulant used (data not shown).

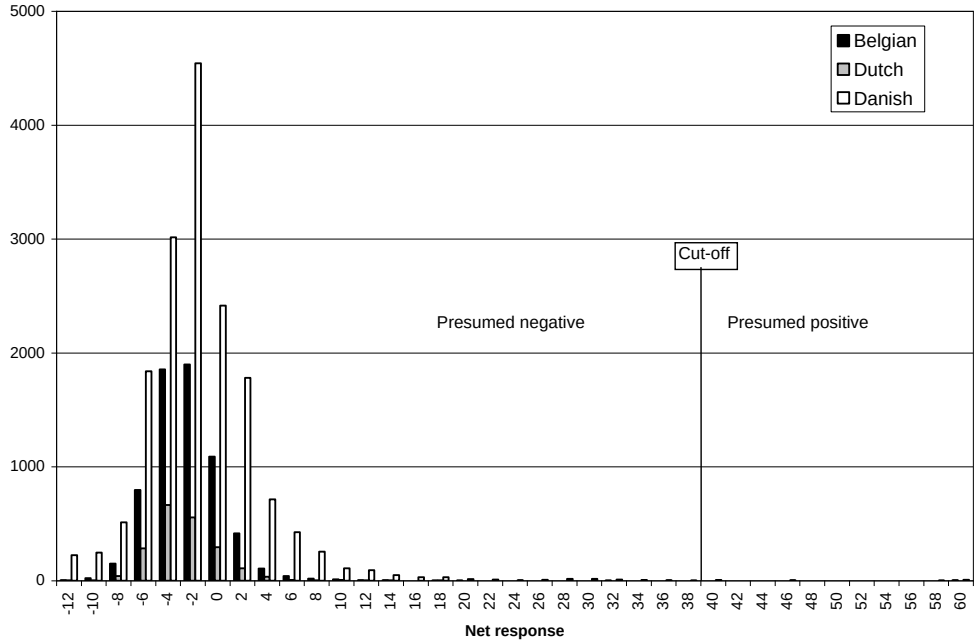

Figure 4: Reactivity of presumed HBsAg negative serum and plasma samples in the Hepanostika HBsAg Ultra assay. The number of samples is presented on the y-axis. The net response (response sample-average negative control) is presented on the x-axis.

HBsAg variants and clinical samples known positive for HBV

One of the major improvements of the Hepanostika HBsAg Ultra assay compared to the Hepanostika HBsAg UF II is the ability to detect most if not all currently known HBsAg variants (19). The ability to detect all clinical relevant HBsAg variants was therefore challenged with 8 clinical (field variants) HBV positive samples and 11 recombinant HBsAg samples with single amino acid substitutions/insertions within the common “a”-determinant (table 2). When testing the diluted HBsAg positive samples in comparison with three other HBsAg screening assays, we found the Hepanostika HBsAg Ultra assay to be the only assay able to detect HBsAg in all samples (table 2). The inability to detect HBsAg variants was most prominent in the ORTHO Antibody to HBsAg ELISA Test System 3, in which only one clinical HBsAg positive sample was detected. The same observation was made with recombinant HBsAg samples; only the wild type HBsAg and two HBsAg variants were detected (G130N and D144H). Although the variant detection of both the ABBOTT Murex HBsAg Version 3 and ABBOTT PRISM HBsAg was better, the Hepanostika HBsAg Ultra assay was found best; as

all clinical HBsAg variants were detected at a level similar to the Hepanostika HBsAg Ultra assay and most of the recombinant HBsAg variants, with the exception of the double mutation variant G145R with a DT insertion at amino acid 122. This sample was not detected in both HBsAg screening assays and only detected with the Hepanostika HBsAg Ultra. Additionally, the ABBOTT PRISM HBsAg was unable to detect the recombinant HBsAg constructs with mutations between amino acid 122 and 124, which explains the lack of detection of the double mutation variant G145R with a DT insertion at amino acid 122.

Table 2: Reactivity of HBsAg variants in four HBsAg screening assays. The reactivity of diluted HBsAg variants in normal human serum in the Ortho antibody to HBsAg ELISA Test System 3 (Ortho), ABBOTT Murex HBsAg HBsAg Version 3 (Murex), ABBOTT PRISM (Prism) and Hepanostika HBsAg Ultra assay (Ultra) is presented as signal over cut off (S/Co). A sample is considered positive when S/Co is greater or equal to 1. Samples that were found reactive are indicated in gray except when the reactivity is clearly less compared to the other assays, than the sample is indicated in bold.

Field variants	**Ortho**	**Murex**	**Prism**	**Ultra**	**dilution**
Ayw3 G145R	0,5	22,8	29,8	21,1	300
Ayw2 P126I/G159A/Y161F	0,5	5,2	6,6	10,6	100
HBsAg, HDV pos	10,6	18,3	26,9	19,7	300
Adw2P120L/T123I/P142S/G145R/ Y161F/S174N/V184A/S204R	0,4	43,4	27,0	53,0	10000
Ayw G145R	0,6	22,6	31,9	22,8	10000
Adw2 G145R	0,2	36,0	46,8	36,5	10000
PCR negative	0,4	4,7	9,1	2,6	300
Ayw3 D144E/G145R	0,5	3,4	5,7	3,2	100000
Recombinant variants					
Wildtype	7,3	11,2	25,5	11,5	1052
122 DT insertion	0,4	3,9	0,3	8,0	147
C124R	0,2	31,1	0,4	41,3	672
G130N	3,8	3,6	8,3	5,0	29
V137Y	0,4	9,0	31,0	30,2	45
C138W	0,2	**1,4**	15,6	7,7	5
K141W	0,4	6,8	9,9	4,1	368
D144H	3,7	5,1	12,8	4,3	144
G145R	0,8	11,5	15,5	8,8	4340
C147S	0,4	6,8	21,0	12,6	17
G145R with 122 DT insertion	0,2	0,5	0,3	1,90	3
Dilution matrix	0,3	0,5	0,5	0,2	

Confirmation of repeat reactive samples

Repeat reactive HBsAg negative samples (n=90) found with the Hepanostika HBsAg Ultra were tested with the Hepanostika HBsAg confirmatory assay to prove its specificity. By testing these 90 HBsAg negative samples, none of the samples were confirmed as HBsAg positive samples. Subsequently, critical HBsAg variants, clinical HBsAg variants, HBsAg subtypes and two high positive HBsAg pools were tested in the Hepanostika HBsAg confirmatory assay. By testing the two high

positive HBsAg pools with the Hepanostika HBsAg confirmatory assay, 90% neutralization was found with 210 IU HBsAg/ml for HBsAg/ad subtypes and 250 IU HBsAg/ml for HBsAg/ay subtypes. Dilution in 9 g/l NaCl confirmed these results (table 3). The HBsAg subtypes were all correctly confirmed positive, as, none of the subtypes showed decreased neutralization in the Hepanostika HBsAg confirmatory assay. Neutralization of all HBsAg variants was possible when the HBsAg variants were reactive close to the detection level of the Hepanostika HBsAg assay. However, a decreased neutralization capacity was observed with some HBsAg variants compared to wild type HBsAg at higher levels of HBsAg (G145R). Nevertheless, all samples were confirmed positive, either when tested undiluted, or 1/11 diluted in 9 g/l NaCl, or after additional 1/100 dilution in 9 g/l NaCl (final dilution 1/1100) when the first test results were inconclusive.

Table 3: Confirmation of HBsAg positive samples in the Hepanostika HBsAg Ultra assay confirmatory assay. The result found after pre-incubation with neutralization solution (Sn) and control solution (Sc) was used to calculate the % inhibition (net response Sc/ net response Sn *100%).

Samples/class	Sc	Sn	% inhibition
recombinant expressed HBsAg variants			
HBsAg/ayw3	2246	20	100%
122DT	475	26	98%
C124R	2617	33	100%
G130N	322	21	99%
C137Y	3000	47	99%
C138W	345	35	95%
K141W	233	17	100%
D144H	246	16	100%
G145R	2595	66	98%
C147S	547	16	100%
G145R+122DT	1628	24	100%
Clinical HBsAg variants			
HBsAg/Ayw3 G145R	2182	40	99%
HBsAg/Adw2 G145R	3000	35	100%
HBsAg/Ayw3 D144E/G145R	2126	18	100%
HBsAg Subtypes			
HBsAg/ayw1	2772	21	100%
HBsAg/ayw2	3000	21	100%
HBsAg/ayw3	2597	21	100%
HBsAg/ayw4	2600	22	100%
HBsAg/ayr	3000	25	100%
HBsAg/adw2	3000	20	100%
HBsAg/adw4	3000	20	100%
HBsAg/adrq+	3000	24	100%
High positive HBsAg samples			
HBsAg/ad 210 IU/ml	3000	234	93%
HBsAg/ay 250 IU/ml	3000	242	93%
diluted 1/11 in 9 g/l NaCl			
HBsAg/ad 2100 IU/ml	3000	297	91%
HBsAg/ay 2500 IU/ml	3000	327	90%

Discussion

The newly developed Hepanostika HBsAg Ultra assay showed good performance compared to other HBsAg screening assays. The Hepanostika HBsAg Ultra screening assay was found to be almost two times more sensitive than the ORTHO Antibody to HBsAg ELISA Test System 3 and has a similar sensitivity when compared to the Abbott/Murex HBsAg version 3. Comparison of sensitivity of the three screening assays with high positive, low positive and seroconversion panels confirmed the high performance level of the Hepanostika HBsAg Ultra screening assay. In tests with the three sample groups we found, in general, equal levels of performance with the Hepanostika HBsAg Ultra and the Abbott/Murex HBsAg version 3, which we found to be more sensitive compared to the ORTHO Antibody to HBsAg ELISA Test System 3 assay. Comparing our results with recently published studies comparing the sensitivity of HBsAg screening assays, places the Hepanostika HBsAg Ultra assay in the top five best CE marked HBsAg screening assays (21). The lower limit of detection of the Hepanostika HBsAg Ultra, 0.04 ± 0.002 WHO IU HBsAg /ml, confirms that the Hepanostika HBsAg Ultra assay is one of the most sensitive HBsAg screening assays (22,23).

The Hepanostika HBsAg Ultra assay is intended for the screening of normal donor populations. Low incidence of repeat reactive HBsAg negative samples is therefore of extreme importance. Testing of 4316 plasma and 4132 sera samples revealed an overall specificity of 99.95 %. Routine testing of Danish donors using the DAVINCI instrument in combination with the Hepanostika HBsAg Ultra assay confirmed this specificity data. The high specificity of the Hepanostika HBsAg screening assay is comparable or better than other published specificity data of HBsAg screening assays (0.18 % - 0.01 %) (24,25). Specificity data depend on the population tested and tends to improve in time (24). Comparison of specificity data between assays is therefore recommended in identical populations and on a site that does not normally uses one of the screening assays.

In this study natural HBsAg variants from infected patients were all detected with wild type sensitivity in three HBsAg screenings assays, only the ORTHO Antibody to HBsAg ELISA Test System 3 was not able to detect the HBsAg variants with mutations in the common “a”-determinant that are not found with the HBsAg subtypes (26). This observation confirms previous findings that reported lack of detection with less frequently found HBsAg variants with the ORTHO Antibody to HBsAg ELISA Test System 3 (13,21). The impact of these HBsAg variants is best illustrated with the seroconversion panel HBV6278 from ZeptoMetrix. The panel is collected from a patient infected with an HBsAg/ayw3 S143L variant and as a consequence of this mutation a delay in detection of 14 days is observed. Recombinant expressed HBsAg variants confirmed the observation of lack of detection with the ORTHO Antibody to HBsAg ELISA Test System 3, in which only three recombinant HBsAg variants out of the in total 11 were detected. The other

HBsAg screening assays detected most of the recombinant expressed HBsAg variants, but with the Hepanostika HBsAg Ultra assay all were detected. The results found with the recombinant HBsAg panel show that the ABBOTT PRISM is poorly or not at all reactive with HBsAg variants with mutations in the 121-124 region. The ABBOTT PRISM uses one capture monoclonal antibody and polyclonal antiHBs for the detection of HBsAg (27). The capture IgM monoclonal antibody was found reactive with the minimal epitope CK/RTC (28,29) confirming our results, showing no reactivity with the 122 DT insertion, C124R and 122 insertion DT / G145R variants. Based on our results it seems that the ABBOTT Murex HBsAg Version 3 uses monoclonal antibodies reactive in different regions in the common "a"-determinant as all single amino acid substitutions were detected, whereas the HBsAg variant with the amino acid substitution/insertion was not detected. Hepanostika HBsAg Ultra assay, designed with two monoclonal antibodies reactive in the common "a"-determinant (amino acid position 120 to 160) and one outside this region showed that all HBsAg variants were detectable. Similar observations were made in a previous study (19).

The ability to confirm reactive samples in a given HBsAg screening assay is a prerequisite for an HBsAg screening assay (25). Therefore, a neutralization fluid based on normal and anti-HBs positive monkey serum was selected and tested with HBsAg negative and HBsAg positive samples. The study showed that all reactive HBsAg negative samples were not confirmed as HBsAg positive samples, whereas all HBsAg positive samples were confirmed positive. The 90 % neutralization level was found at 210 to 250 IU/ml, which means that HBsAg positive samples with a titer of 210 IU/ml will be neutralized without pre-dilution and that samples with higher HBsAg titers need to be diluted below this level. The ability to neutralize critical HBsAg variants showed that the neutralization level was lowered, but that still all samples could be neutralized either or not after additional dilution.

In conclusion: the Hepanostika HBsAg Ultra assay is the first HBsAg screening assay designed and shown to detect variants having extreme changes in the common "a"-determinant. We confirmed published data that other diagnostics assays fail to detect certain HBsAg variants (12–17). The pressing need for a screening assay, which is capable of detecting all HBsAg variants, was recently reported in consensus meetings (7,30). By introducing the new Hepanostika HBsAg Ultra assay and confirmatory assay we can answer to this need

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Chapter 6: Epitope Mapping of Neutralizing VHH Antibody against Hepatitis B Virus S-Protein

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Abstract

Hepatitis B virus (HBV) infections can be prevented with anti-HBs antibodies against the small S-protein. Recently single-domain antibodies of Llamas (VHH) that recognize the small S-protein of HBV were isolated. We determined the epitopes of the most promising VHH on HBsAg and showed importance of the c-terminal part of the S-protein as well as correct conformation of the antigenic loop for the recognition of the S-protein by VHH S5. When tested in the HDV infection model we found equal neutralization with VHH S5 compared to the best neutralizing anti-HBs monoclonal reported. Surprisingly a bi-head of two non-neutralizing VHH was as active in neutralization as the conventional masking antibody C20-2. The small size of the VHH compared to previously used antibodies shows for the first time a direct relation of a small, cross linked cysteine epitope of the S-protein and a yet to be identified cellular receptor.

Introduction

Hepatitis B virus (HBV) is a major cause of acute and chronic liver disease worldwide. Despite the introduction of universal vaccination against Hepatitis B, persistent HBV infection is still a serious problem. Globally it's estimated that 350 million carriers exist of whom many will develop serious liver diseases, including hepatocellular carcinoma (13). HBV infected patients can be super infected with a satellite virus called hepatitis Delta virus (HDV). The assembly of both HBV and HDV depend on interaction between the surface antigen and the HBV nucleocapsid or ribonucleoprotein in the case of HDV (1, 24, 32). Because the exterior of HBV and HDV particles are identical, a study of the HBV envelope proteins functions at viral entry can be conducted using the HDV model (25, 27). Natural self-limited HBV infection raises protective antibodies mostly directed against the major antigenic "a"-determinant of Hepatitis B surface antigen (HBsAg), which is present in all serotypes (4). The antibodies prevent new infections and can be used for passive immunotherapy against HBV infection in cases of accidental needle stick injuries, for liver transplant patients and to prevent vertical transfer from mother to child (14, 21). Antibodies against the small S-protein are thus directly related to prevention of HBV infections either via a direct or indirect neutralization of the HBV virion. Recent studies have shown that monoclonal anti-HBs antibodies can prevent infection (20) and showed involvement of the antigenic

loop of the surface antigen with viral entry. Infection with HBV can be prevented by anti-HBs antibodies (19) although the mechanism(s) of action is still unclear, it seems that specific populations of anti-HBs can block a currently unknown S-protein related receptor (20, 31). The differential ability to block viral entry has been shown in the HDV infection model (20) where anti-HBs monoclonal A1.2 has been shown to prevent infection efficiently while monoclonal C20-2 could only block the infection at high IgG concentrations. In other studies anti-HBs antibodies have effectively prevented or reduced HBV infections (6, 10) again showing involvement of the small S-protein in viral entry. Still due to the relative large size of IgG's (18) it's able to mask a relative large part of the surface of HBV making it hard to pinpoint the true region involved. Recently single domain antibodies (VHH's) against HBsAg were successfully generated from the variable heavy domain of the heavy-chain antibodies of the Camelidae (22). These antibodies are of interest for their ability to recognize structures that are not accessible by conventional antibodies (3, 8). Compared to IgG, VHHs have a more simple structure, they are smaller in size (11-15 kDa) and they are more hydrophilic. This results in improved solubility and expression in heterologous systems, making them highly suitable for prophylactic products (11, 33).

In this study VHH's generated and selected against HBsAg were tested on ability to neutralize HBV using the HDV infection model (25, 27). Virion neutralization provided detailed evidence that a small part of the S-protein is involved as a secondary cellular binding protein.

Material and Methods

VHHs

The VHHs (S1-S5) used in this study were selected previously using recombinant HBsAg and were kindly provided by Serruys et al. for this study (22).

VHH-phage ELISAs

Maxisorp plates were coated overnight with 1 µg/ml rHBsAg at room temperature and blocked with PBS 1% casein for 2 hours. Supernatant of the mini cultures (obtained as described above) were added for 2 hours; VHH-GenIII supernatant was diluted 3-fold, while VHH-phage supernatant was diluted 10-fold. VHH-GenIII was detected using mouse anti-myc, followed by anti-mouse horseradish peroxidase (HRP) conjugate. To detect VHH-phage particles, mouse anti-M13-HRP conjugate was used. For both ELISA's OPD H_2O_2 0.05 M citrate-phosphate buffer (pH 4) was added for 30 minutes. After the addition of 1 N H_2SO_4, the OD was read at 405 nm.

Construction of bivalent VHHs

Bivalent VHH were constructed by cloning a PCR amplified VHH fragment containing a flexible $(GGGGS)^2$ hinge of 10 amino acids in length after the VHH fragment in the pAX004 expression vector. The VHH PCR fragment and pAX004 containing VHH expression vector were both digested with BstEII and NotI, purified, ligated and transformed to TG1 cells. Clones with the correct length were selected and send for confirmation of the sequence.

Purification of monomeric and bivalent VHHs.

Production of soluble VHHs were done in the non-suppressor *E. coli* strain WK6. Cells were grown at 37°C in 250 ml Terrific Broth-0.1% glucose-100 µg/ml ampicilline (Sigma) until an absorbance between 0.5 and 1 was reached. 1 mM IPTG and 5 mM $MgSO_4$ were added. After 4 hours at 37°C, cells were harvested and the periplasmic proteins were extracted by osmotic shock with 50 mM NaH_2PO_4-300 mM NaCl. The VHHs were purified by Ni^{2+}-affinity chromatography (Talon Metal Affinity resin, BD Biosciences). Presence of aggregates was detected by subjecting the purified VHH fragments to a HiPrep 16/60 Sephacryl S-200 HR column (GE-Healthcare, the Netherlands).

VHH ELISA.

Maxisorp plates were coated with pHBsAg (1 µg/ml in PBS) and blocked with PBS-1% BSA. After three washes with PBS-0.1% Tween20, VHHs at concentrations indicated were added to the wells and incubated for 2h at room temperature. After three washes, mouse anti-myc was added, followed by anti-mouse HRP conjugate (Sigma). HRP-activity was determined with tetramethylbenzidine (TMB) substrate. After the addition of 1 N H_2SO_4, the OD was read at 450 nm.

Biotinylation of VHHs.

The purified VHH's were biotinylated using 10 mol excess of NHS-Biotin (Pierce) in PBS. After 2 hours incubation at room temperature the reaction was quenched with 100mM Glycine. The biotinylated VHH's were tested in the VHH ELISA using streptavidin-HRP as conjugate (Roche). HRP-activity was determined with tetramethylbenzidine (TMB) substrate. After the addition of 1 N H_2SO_4, the OD was read at 450 nm.

Cross inhibition of VHHs.

Maxisorp plates were coated with pHBsAg (1 µg/ml in PBS) and blocked with PBS-1% BSA. After three washes with PBS-0.1% Tween20, VHHs and biotinylated VHH’s were mixed at concentrations indicated and added to the wells and incubated for 2h at room temperature. After three washes, streptavidin-HRP was

added to the wells. HRP-activity was determined with tetramethylbenzidine (TMB) substrate. After the addition of 1 N H_2SO_4, the OD was read at 450 nm.

Epitope mapping of VHHs.

The sequence described for HBsAg of subtype adw2 (genotype A) and ayw2 (genotype D) were used to synthesize 30-mer peptides (Pepscan bv. Lelystad, the Netherlands). Starting from amino acid 99 of the small S-protein and shifting one amino acid, even peptides were synthesized based on the adw2 sequence and odd peptides on the ayw2 sequence. Thus the first peptide was based on the adw2 sequence and the second on the ayw2 subtype. The 30-mer peptides were synthesized via a new peptide library technology developed at Pepscan bv. Briefly, the first 14-mer segment is synthesized on the surface of chip and later the second segment is coupled to the first half via a non-peptide bound linker. Due to this procedure amino acids 14 and 15 within the 30-mer are artificial and in peptides where these amino acids are involved in antibody antigen interaction, a negative result is expected. The VHH antibodies were tested at Pepscan bv according to standard procedures (5).

Reactivity of VHH antibodies with HVD bearing single amino acid substitutions

VHH's were coated at 5µg/ml in 50mM carbonate solution pH=9.6 on maxisorp plates (NUNC) overnight at room temperature. The next day, the plates were washed three times with PBS/tween and post-coated with 0.5% Casein in PBS for 2h. The specific reactivity of the coated VHH with HDV particles bearing amino acid substitutions (Q101A, G102A, M103A, L104A, P105A, V106A, P108A,L109A, I110A, P111A, G112A, S113A,S114A, T115A, T116A, S117A, T118A, G119A, P120A, C121S, R122A, T123A, C124S M125A, T126A, T127A, A128G, Q129A, G130A, T131A, S132A, M133A, Y134A, P135A, S136A, C137S, C138S, C139S, T140A, P142A, S143A, D144A, G145A N146A, C147S, T148A, C149S, I150A, P151A, I152A, P153A, S154A, S155A, W156A, F158A, G159A, K160A, F161A, L162A, W163A, E164A, W165A, S167A, R169A, F170A, W172A) in the antigenic loop was assessed by testing diluted HDV particles in the coated wells. Detection of bound viral particles was achieved by adding 100 µl/well of a 1/1,000 dilution of rabbit anti-pre-S2 antibody (R257) in PBST–10% FBS, followed by incubation at 37°C for 2 h. The plates were washed three times with 200 µl of PBST/well. Then, 100 µl/well of a 1/2,000 dilution of HRP-labelled anti-rabbit antibody in PBST–10% FBS was added, and the plates were incubated at 37°C for 1 h. After five washes with 200 µl of PBST/well, 100 µl of Ultra TMB HRP substrate (Pierce)/well was added, followed by incubation at room temperature for 15 min. The color reaction was stopped with sulphuric acid. The optical density was measured at 450 nm with an ELISA plate reader.

In vitro infection assays.

HepaRG cell cultures were treated with 2% dimethyl sulfoxide for 2 weeks prior to inoculation with HDV particles (7). Inocula consisted of culture fluids collected from HuH-7 cells at days 5, 7, and 9 post transfection with HDV (genotype A-E), which were pooled and clarified by centrifugation at 5,000 x g for 30 min at 4°C. HepaRG cells (3 x 10^5 cells/20-mm-diameter well) were exposed to 100 genome equivalents (GE) of HDV virions mixed with variable amounts VHHs or IgG as indicated. After 16h exposure in the presence of 5% polyethylene glycol 8000, the supernatant was removed and replaced by new medium. Cells were harvested at day 7 post exposure for measurement of intracellular HDV RNA that served as a marker of infection. HDV RNA signals were detected by Northern blot analysis using a ^{32}P-labeled RNA probe and quantified using a phosphor imager.

Results

Cross inhibition of VHHs.

Prior testing the VHH on cross inhibition, we tested all five VHH's on plates coated with HBsAg/ad or HBsAg/ay and found that all VHH's except VHH S1 were equally reactive on both types of HBsAg (Figure 1).

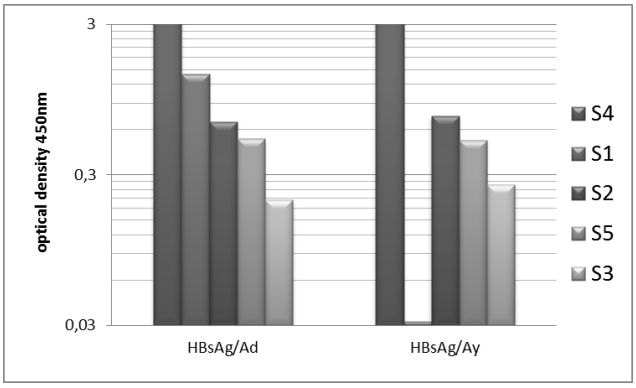

Figure 1: Reactivity of VHH's on plates coated with HBsAg/ad and HBsAg/ay. The VHH were diluted to 0.1µg/ml and the found reactivity is expressed (optical density at 450nm). The VHH were sorted on reactivity with the HBsAg/ad coated plate (S4 best, S3 least reactive VHH).

As the VHH S1 was poorly reactive with HBsAg/ay we chose to proceed with the cross inhibition studies with HBsAg/ad coated plates. Hereto we labeled the five selected VHH's with biotin and tested 0.5 µg/ml of the labeled VHH's mixed with 10µg/ml unlabeled VHH's on plates coated with pHBsAg/ad in order to determine epitope similarity. As expected all reactivity (complete inhibition) was gone when biotinylated VHH was mixed with a matching unlabeled VHH (Table 1). Remarkably

VHH S4 could inhibit the binding of all VHH's except for VHH S3; VHH S3 seems to recognize a unique epitope as it is unable to reduce the binding any of the other 4 VHH's. This made S3 an interesting candidate for the generation of a bi-specific bi-head VHH. Based on the cross inhibition study we can conclude that VHH S4 and S5 recognize more or less the same epitope, both VHH's are inhibited by the same VHH's (S1, S2) and S4/S5 is able to inhibit the binding of S5 and S4. The inability of S5 to efficiently interfere with the binding of S4 might be explained by the lower affinity as observed in previous experiments using the VHH (22).

Table 1: Cross inhibition of VHH's. 0.5µg/ml biotinylated VHH was mixed with 10µg/ml VHH (unlabeled) and tested on HBsAg/ad coated plates. Bound biotinylated VHH was detected with streptavidin-HRP. Complete inhibition is indicated by ++, almost complete by +, marginal by +/- and none by -.

Biotinylated	Unlabeled VHH 10µg/ml				
VHH 0.5µg/ml	S1	S2	S3	S4	S5
S1	++	-	-	+	+/-
S2	-	++	+/-	++	+
S3	-	-	++	-	-
S4	-	-	-	++	+/-
S5	-	-	-	+	++

Epitope mapping of VHH S5

The binding epitope of VHH S5 was investigated by testing purified S5 VHH fragments on peptides covering the c-terminal part of the small S-protein of HBV. We found specific binding of the VHH with the c-terminus of the S-protein, the first reactive peptide was 188-SPTVWLSVIWMMW&&GPSLYSILSPFLPLL-216 and the last reactive peptide 193-LSVIWMMWYWGPSL&&ILSPFLPLLPIFFCL-222 (figure 2). The reactivity was slightly better for the peptides based on the adw2 (genotype A) sequence compared to the reactivity with peptide based on the ayw2 (genotype D) sequence. The expected dip in reactivity due to the linker passing through the binding epitope was not observed and made us believe that the minimal sequence reactive sequence involved with VHH S5 binding is 193-LSVIWMMW-200-XXXXXX-207-ILSPFLPLL-216, or only the c-terminal part of the two sequences 207-ILSPFLPLL-216.

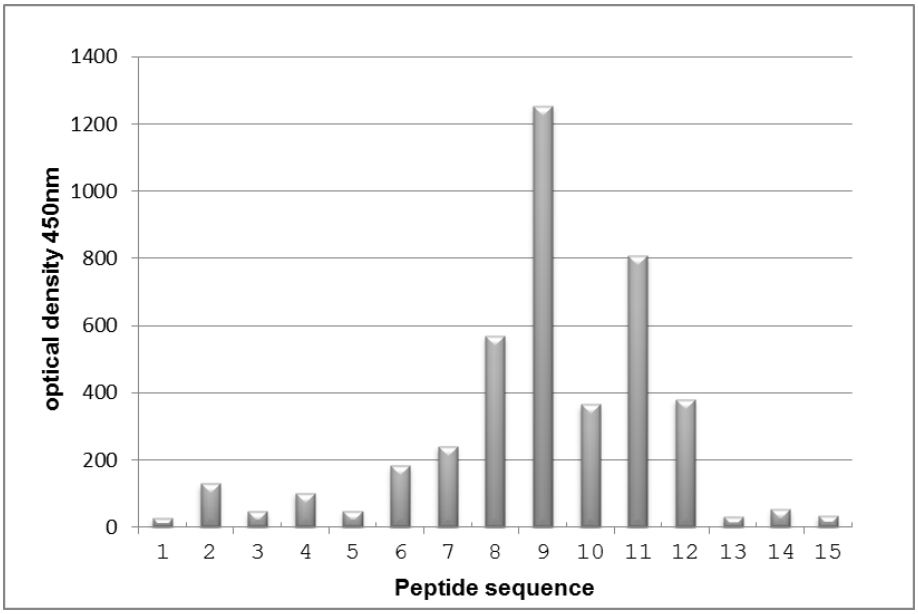

Figure 2: Pepscan analysis of VHH S5 on overlapping 30-mer peptides from residues 188–225. The peptide numbers are indicated below each bar representing the following sequences; 1:QWFVGLSPTVWLSA&&MMWYWGPSLYSIVSP, 2:WFVGLSPTVWLSVI&&MWYWGPSLYSILSPF; 3:FVGLSPTVWLSAIW&&WYWGPSLYSIVSPFI; 4:VGLSPTVWLSVIWM&&YWGPSLYSILSPFLP; 5:GLSPTVWLSAIWMM&&WGPSLYSIVSPFIPL; 6:LSPTVWLSVIWMMW&&GPSLYSILSPFLPLL; 7:SPTVWLSAIWMMWY&&PSLYSIVSPFIPLLP; 8:PTVWLSVIWMMWYW&&SLYSILSPFLPLLPI 9:TVWLSAIWMMWYWG&&LYSIVSPFIPLLPIF; 10:VWLSVIWMMWYWGP&&YSILSPFLPLLPIFF; 11:WLSAIWMMWYWGPS&&SIVSPFIPLLPIFFC; 12:LSVIWMMWYWGPSL&&ILSPFLPLLPIFFCL; 13:SAIWMMWYWGPSLY&&VSPFIPLLPIFFCLW; 14:VIWMMWYWGPSLYS&&SPFLPLLPIFFCLWV; 15:AIWMMWYWGPSLYS&&SPFIPLLPIFFCLWV. The found reactivity (optical density at 450nm) is expressed.

The region(s) 193-LSVIWMMW-200-XXXXXX-207-ILSPFLPLL-216 was already recognized by others in relation to HBsAg/antibody binding (2, 12), but never as a sole binding epitope for antibodies. To confirm our observations we tested the VHH S5 coated on µplates with HDV particles bearing single amino acid substitutions in the major antigenic loop (AGL) of the virus (20). Surprisingly, we found that the reactivity of the VHH S5 was affected by many mutations in the AGL (figure 3), suggesting the involvement of this region in the antibody binding. All the tested cysteines and K141A and I152A abolished the binding.

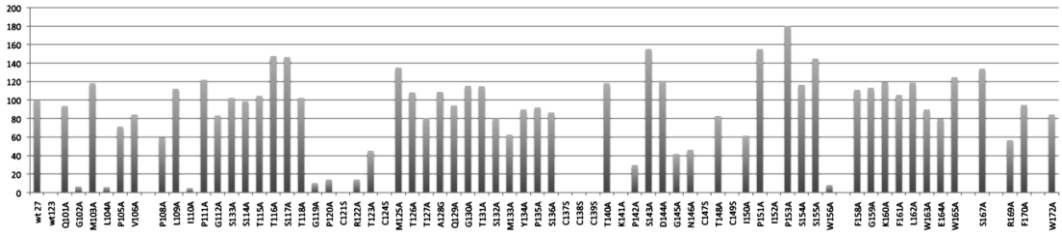

Figure 3: Reactivity of VHH S5 with HDV particles bearing amino acid substitutions in the AGL. The histogram is showing the reactivity (optical density) with S5 with the different mutated HDV particles. Mutations are substitutions of serine for cysteine residues or alanine for non-cysteine residues as indicated below each reactivity bar.

As with reported monoclonal antibodies (2, 12) the VHH S5 is reactive with peptides in the c-terminal part of the S-protein, but also dependent on the correct confirmation of the AGL. Whether the antibody is directly involved in binding to the AGL or is affected by conformational changes due to the mutations needs to be established.

Neutralization of HDV by VHH's.

HDV infection can be studied in HepaRG cells and its infection can be prevented in presence of anti-HBs or anti-PreS1 antibodies (6, 26). To study the neutralization capability of the VHH, different concentrations VHH were tested in the HDV infection model (Figure 4). We found that the infection of HDV could be prevented by the VHH S5 in a dose dependent way, which is quite remarkable as this VHH was one of the least reactive VHH during screening. The epitope similarity of S4/S5 observed in the cross inhibition studies was confirmed in the neutralization experiments although the neutralization efficiency of VHH S4 compared to S5 was at least 10 times less but better than the reference antibody with C20-2 (anti-HBs (9)). The neutralization capacity of VHH S5 was less compared to the reference good neutralizing antibodies MA18/7 (anti-PreS1 (9)) and A1.2 (anti-HBs (15)). The lower affinity of the VHH as observed by Biacore analysis (22) and the monovalency of the VHH's could explain the lower potency of the VHH compared to the monoclonal antibodies tested as reference, that normally have K_D of 10^9 M or higher (28) while VHH's described here have K_D of 10^8 M and lower (22). The VHH's S1, S2 and S3 were unable to prevent infection and could be used to model the influence of size in viral neutralization. To be able to answer this we constructed a bihead VHH consisting of VHH S3 linked to S1 via a 10amino acid long flexible spacer. Improved neutralization capability of VHH S5 was tested by cloning the S5 VHH head to head with a 10 amino acid flexible spacer between the two copies (bihead S5). The bihead S5 and S3-S1 used in the neutralization experiment with HDV were able to prevent the HDV infection. The bivalent VHH S5 was able to prevent the HDV infection comparable to the control anti-HBs A1.2, while bi-specific VHH S3-S1 was equal effective when compared with the reference

antibody with C20-2 (Figure 4). To show broad application of the VHH fragments with different HBV genotypes we tested the neutralization capability of the VHH fragments with 5 HBV genotypes / 6 subtypes in the HDV infection model. Again we observed pronounced neutralization activity with VHH S5 while S4 could only reduce infection of the genotype D/ayw3 type and E/ayw4 type. The other VHH's could not reduce the infectivity of different HDV types tested (figure 4).

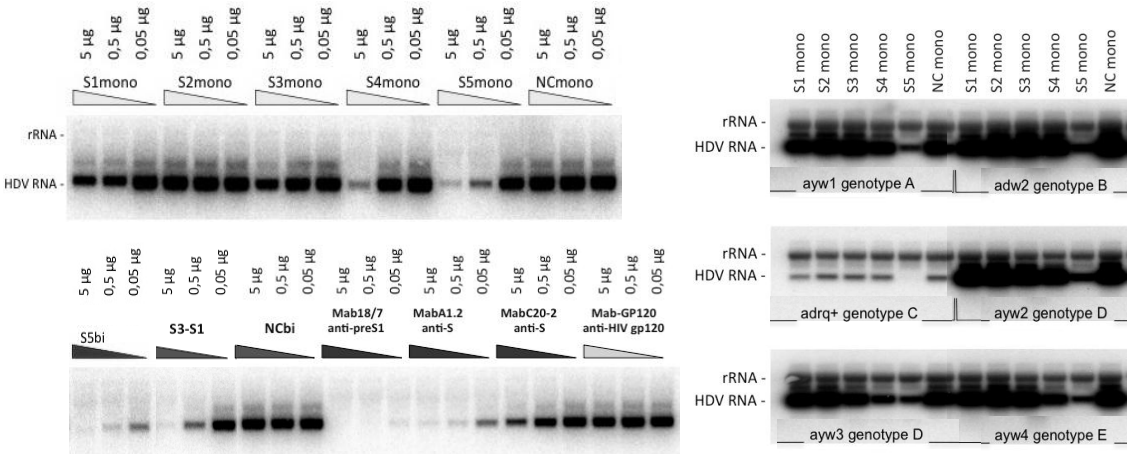

Figure 4: A: RNA blot hybridization analysis of HDV RNA extracted from HepaRG cells exposed to HDV particles (genotype D/ayw3). 10^6 cells were exposed to 100 MOI HDV particles using inocula in presence of 5% PEG that had been mix without or with antibodies specific for HBV envelope protein VHH S1-S5, bi-head constructs (S5bi, S3-S1 & NCbi), PreS1 (MA18/7) and S (A1.2, C20-2) and as negative control with a monoclonal specific for HIV envelope protein gp120. Total cellular RNA extracted from hepatocytes harvested 7 days after inoculation was analyzed for the presence of genomic or anti-genomic HDV RNA. The RNA genomes were detected after agarose gel filtration and hybridization (indicated by HDV RNA). The amount of antibody used is indicated above each lane. B: Repeated with Genotypes A till E neutralized with 0.5 µg VHH S1-S5 & NC. The presence ribosomal RNA (rRNA) is indicated for reference.

Discussion

We present for the first time a broad neutralizing VHH antibody reactive against the small S-protein of HBV with the potential to be used as prophylactic agent against HBV. The bi-head VHH revealed equal potency compared to best neutralizing anti-HBs antibodies and was able to prevent infection of at least 5 different subtypes. The small size of the VHH proofs involvement of a restricted region on the small S-protein that is involved in binding with a potential second cellular receptor. We tested five VHH's reactive in different regions of the small S-protein. The VHH were previously described and shown to retain HBsAg particles when the expressed in the endoplasmic reticulum as intrabodies (22) and let us believe that the VHH's

could be an interesting alternative source of antibodies that neutralizes HBV and directly show involvement of the small S-protein in viral entry.

To challenge this idea we first characterized the antibody binding properties with pHBsAg/ad and pHBsAg/ay coated plates and confirmed previous experiments (22) when pHBsAg/ad coated plates were used. We noted a dramatic decrease in reactivity when the VHH S1 was tested on plates coated with HBsAg/ay, the VHH S1 lost almost all reactivity. The type specificity of this antibody was confirmed by the intrabody experiment with HBsAg/ayw where the VHH S1 was poorly reactive (22). The other VHH's were all reactive with both HBsAg subtypes/genotypes with equal affinity and thereby in theory suited to prevent infection of different HBV strains.

The binding epitope of VHH S5 was determined by testing the VHH on peptides derived from HBsAg/adw2 and ayw2. We found specific reactivity in the c-terminus of the S-protein 193-LSVIWMMW-200----207-ILSPFLPLL-216. However to our surprise, the reactivity of S5 with mutated HDV particles revealed involvement of all cysteines in the AGL and involvement of K141A and P152A showing that the binding of S5 is dependent on amino acids within the AGL. Possibly the c-terminus of the S-protein is folded in close proximity of the AGL and changes in the AGL could also affect the folding of the c-terminal part of S-protein.

In our *in-vitro* studies two VHH's (S4 and S5) could prevent HDV infection when 5µg VHH was incubated with 100 MOI HDV, while VHH S5 was even effective at 0.5 µg, which is quite remarkable as this VHH had a lower affinity for HBsAg compared to S4 (Table 1, (22)). Based on cross inhibition experiments and neutralization experiments we can conclude that the VHH S4 and S5 recognize a similar epitope on the S-protein, with S5 closest to the region involved in viral entry. A bi-head construct with two non-competing VHH fragments was constructed to test for virion masking effects in neutralization experiments. Surprisingly, we found that the bi-head construct with two not neutralizing fragments we can reduce infectivity similar to the level observed with the C20-2 anti-HBs monoclonal, but less compared to A1.2 and 18/7 monoclonal antibodies. Based on these observations we believe that C20-2 masks the virion while A1.2 and 18/7 are able to block viral entry specifically, as observed with VHH S5. The broad neutralization capacity of S5 with different HBV genotypes was confirmed in the HDV model experiment with 4 different genotypes and 5 substypes. In all cases S5 neutralized the infection equal to the level observed in the initial neutralization experiments. The VHH S5 tested as bivalent binding molecule resulted in a dramatic increase of neutralization capacity, at least 10-fold increase in potency is observed. The increase in potency can be explained by the high K_{off} (22). The high K_{on} observed with the monovalent VHH results in fast binding, but due to the high K_{off} it results in a quick release of the VHH from HBsAg. In case of the bi-head, with two binding

parts, it results in fast binding of both binding regions and although the different parts of the VHH release easily, the whole molecule remains close to HBsAg and enables its renewed binding. Similar observations were noted previously with recombinant antibodies also showing increased affinity with monovalent recombinant antibodies with high K_{off} when used as multivalent recombinant antibodies (16). The unique pinpoint recognition of the monovalent VHH S5 clearly indicates that a small part of the S-protein is involved in the viral entry either by binding to a primary or secondary receptor on hepatocytes.

The possible involvement of the C-terminal part of the S-protein in viral entry is strengthened by the observation that the human monoclonal antibody f4-7b that is able to reduce infectivity (10). This antibody has been confirmed reactive in the c-terminus of the S-protein (17). The importance of the c-terminal sequence in viral entry is further supported by observations that DHBV anti-HBs neutralize DHBV infections (29). In DHBV the AGL is absent and the anti-DHBs response is thus directed against homologue sequences corresponding to the c-terminus of HBsAg. The involvement of these residues in viral entry either direct or indirect has been proposed by others (20).

As today only computer aided models are available (2, 23, 30) we are not able to proof this hypothesis. Therefore we decided to plan experiments attempting to co-crystallize the VHH S5 with HBsAg or HBsAg fragments in an attempt to clarify the true binding region of S5.

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Chapter 7: Computer aided modeling of the C-terminal part of the S-protein

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Abstract

The small S-protein of the hepatitis B virus (HBsAg) can be divided in four different functional parts, the first part is a membrane spanning region, followed by a second hydrophilic region interacting with the core particle flanked by again a membrane spanning region, which is followed by a hydrophilic and a hydrophobic sequence known to be on the outside of the virion. Computer aided modeling of the last C-terminal region in conjunction with previously described neutralizing VHH, revealed a dimer structure bringing together key experiments that were conducted to unravel the known complex network of sulfur bridges present in the C-terminal part of the S-protein. We show in our model that 12 of the in total 16 cysteine residues present in the dimer structure are positioned in such a way that disulfide bridging is possible between any of the 12 cysteines, with a central position of the three cysteines (aa137-139). The found structure matched well with the published topology data and with studies using antibodies or site directed mutagenesis. The C-terminal hydrophobic region was analyzed separately and shown to have structural homology with Arfaptin 2, a BAR domain protein. In conclusion we present for the first time an 'ab initio' structural model of the C-terminal fragment of the S-protein. The model indicates a dimeric protein fragment embedded in the lipid membrane placing the “a”-determinant on top of the membrane structure and the remaining C-terminal fragment peripherally stabilized via tryptophan and leucine residues. We propose that the HBV infection is initiated by binding of the pre-S1 followed by non-specific membrane fusions mediated by the C-terminus of the S-protein shifting from a I-BAR to a F-BAR conformation.

Introduction

Hepatitis B virus (HBV) is the major cause of hepatitis in humans. HBV infection in humans results in 5-10% in a chronic infection, eventually leading to the development of liver cirrhosis or liver carcinoma. It is estimated that there are 350 million people chronically infected with HBV (1). During a hepatitis B infection the liver secretes complete virions (Dane particles) and non-infectious 22nm particles with small quantities of filaments (100-200x22nm), all carrying the viral envelope (2). The viral envelope is a complex structure composed of cellular lipids and three

related viral proteins, the large (L), middle (M), and small (S) surface proteins (3–5).

Transfection of the gene coding for the small S-protein in cell lines have shown that 22nm particles are secreted (3,6–9) and that these particles have comparable morphological structure and immunological behavior compared to the native 22nm particles (3, personal observations). After translation, the S-protein is inserted into the membrane of the endoplasmic reticulum (ER) where it is arrested to form dimers in the presence of protein disulfide isomerase (PDI) (10,11). After accumulation, about 48 dimer S-proteins are directed to the early Golgi compartment where the S-proteins maturates to form 22nm particles (10). Here some of the in total 14 cysteines present in the S-protein crosslink to form a highly stable 22nm particle (4,10). The budding process involves substantial reorganization of the membrane lipids with help of chaperones (5,10,12,13). After budding, the 22nm particle leaves the cell via the constitutive pathway of secretion.

The S-protein contains three hydrophobic domains located at amino acids (aa) positions 11 to 28, 80 to 98, and 169 to 226 (4,4,11,14) that separate two hydrophilic regions aa 29 to aa 79 and aa 99 to aa 168 (Figure 1). Analysis of mutated and deleted forms of the S-protein have shown that both the entire first and second hydrophobic domains serve an essential function in determining protein topology (6,7,15,16). The first region contains a N-terminal signal sequence, but lacks a cleavage sequence (15,16). The second domain anchors the protein in the lipid bilayer (15,16). This results in a topology where the first hydrophilic region has a cytoplasmic orientation and the second hydrophilic region is oriented to the lumen (i.e. external in the mature particle). The cytoplasmic orientation of the first loop is confirmed by the observation that the sequence is accessible to proteases when anchored in the ER and is inaccessible by proteases in the mature particle (4,14,16,17). Furthermore, a potential N-glycosylation site (NXS/T) at amino acid 59 present in various subtypes of HBV is left unused. The orientation of the second hydrophilic region is confirmed by the observation that the sequence is N-glycosylated and the region is accessible to the immune system (18–20). The last hydrophobic region of the S-protein is probably exposed to the lumen since a linear epitope at amino acid positions 178 to 186 is accessible to the immune system and can be used to detect HBV and 22nm particles (21). Epitope mapping utilizing (filamentous phage) peptide libraries showed that monoclonal antibodies and VHH recognizing conformational epitopes on the 22nm particle, probably interact with amino acids in the last hydrophobic region (20,22, Roosmalen et al. to be published) again indicating that this region of the S-protein is surface exposed.

Recently new data on HBsAg particles studied by cryo-electron microscopy confirmed the dimer building block, but were not detailed enough to determine the

complex structure of the second hydrophilic loop (“a”-determinant) (23,24). Here we describe for the first time by *ab initio* modeling, a quaternary structure for the “a”-determinant of the HBV surface protein complexed with a neutralizing VHH antibody. The various features of the proposed model are discussed in the light of published topology data presented with antibodies and site directed mutagenesis.

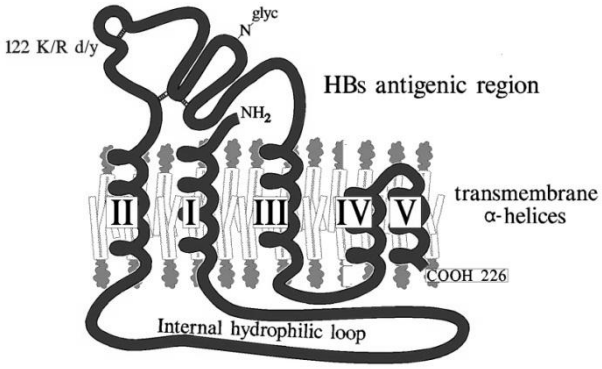

Figure 1: Proposed topology of the small envelop protein by Stirk (25), adapted by Gerlich

Materials and Methods

VHH

The VHH S5 used for our modeling studies was selected against HBsAg particles as described in our previous work (26). We selected VHH S5 for its potent neutralizing features that should recognize structures in the small S-protein involved in viral entry (Roosmalen et al. to be published).

Sequence analysis

Ten published HBsAg sequences found in different species were selected and aligned to determine conserved regions. We used the following sequences: arctic ground squirrel (gi|939698), crane (gi|26800786), duck (gi|401375), gibbon (gi|82016665), gorilla (gi|82039700), orangutan (gi|82026688), Human (gi|138799), snow goose (gi|4530356), stork (gi|17932896), woolly monkey (gi|82016122).

Tertiary structure prediction

Today no template structure is present for the 226-amino-acid-long S-protein (27). Therefore, HBsAg fragments artificially fused using a $(GGGS)^{10}$ linker between each protein chain were submitted for structure predictions using '*ab initio*' or '*de novo*' modeling (28). In this study, we used the I-TASSER method, a protein structure modeling approach based on an algorithm that consists of consecutive steps of threading, fragment assembly, and iteration to obtain a structure with the lowest energy as described previously (28–30). All structure predictions were done separately using individual I-TASSER queries, and visualized using PyMOL (31).

RESULTS and DISCUSSION

Currently there is consensus that the major building block for HBsAg particles consist of dimers, folded together to a still unknown structure (10,11). The main structural questions are related to the disulfide bridges and position of helixes. Alignment of Hepatitis B virus sequences collected from different species revealed a high degree of similarity (Figure 2). The conserved regions in the S-protein with main functions for folding and membrane transitions are indicated.

```
                         HSHSHSHSHSHSHS     CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCHSHSHSHSHSHSHSHSH          AAAAAAAAAA
Gibbon            MENITSGFLGPLLVLQAGFFLLTKILTIPQSLDSWWTSLNFLGGAPVCPGQNSQSPTSNHSPTSCPPICPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLPVCPLLPG..SS   114
Human             MENITSGFLGPLLVLQAGFFLLTrILTIPQSLDSWWTSLNFLGGttVClGQNSQSPTSNHSPTSCPPtCPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLPVCPLiPG..SS   114
Gorilla           MEsITSGFLGPLLVLQAGFFLLTKILTIPQSLDSWWTSLNFLGGAPVClGQNSQSPTSNHSPTSCPPICPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLPVCPLiPG..St   114
Orangutan         MEsIsSGFLGPLLVLQAGFFLLTKILTIPQSLDSWWTSLNFLGGAPVCPGQNSQSlTSNHSPTSCPPICPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYrGMLPVCPLLPG..tt   114
Woolly monkey     MditsSGFLGPLLaLQAvFFLLTKILTmPQSLDSlWTSLNFLGGtPaCPGlNSQSPTSsHSPTcCPPtCPGYRWMCLRRsIIFLFILLLCLIFLLVLLDYQGMLPVCPLLPtvtgt   116
ground squirrel   M..spSGlLGlLagLQvvyFLwTnILTIaQSLDwWWTSLsFpGGiPeCtGQNlQflTckHlPTSCPPtCnGfRWMyLRRFIIyLlvLLLCLIFLLVLLDwkGlLPVCPiqP....S   110
Stork             MgasfgGiLagLigLlvGFFLLTKILeIlrkLDwWWiSLsspkekmlCafQstghqTSpHyvgSCPwgCPGflWtyLRlsIIFLlILLvaaglLf.Ltenk..............   100
Crane             MsstfgGiLagLigLlvGFFLLiKILeIlrkLDwWWiSLsFpkermqCtfhdtgaqiSpHyvgSCPwgCPGflWtyLRlFIIFLlILLvaaglLy.LtDnm..............   100
Duck              MsgtfgGiLagLigLlvsFFLLiKILeIlrrLDwWWiSLsspkGkmqCafQdtgaqiSpHyvgSCPwgCPGflWtyLRlFIIFLlILLvaaglLy.LtDng..............   100
Snow goose        MsgtfgGiLagLigLlvsFFLLiKILeIlrkLDwWWiSLsspkGkmqCafpdtgaqiSpHyvgSCPwgCPGflWtyLRlFIIFLlILLvaaglLy.LtDng..............   100

                  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA    HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
Gibbon            TTSTGPCRTCTITAQGTSLYPSCCCTKPSDGNCTCIPIPSSWAFAKFLWEWASVRFSWL.SLLAPFVQWFAGLSPTAWLLVIWMIWYWGPNLYNILNPFIPLLPIFFCLWVYI   226
Human             TTSTGPCRTCmtTAQGTSmYPSCCCTKPSDGNCTCIPIPSSWAFgKFLWEWASaRFSWL.SLLvPFVQWFvGLSPTvWLsVIWMmWYWGPsLYsILsPFlPLLPIFFCLWVYI   226
Gorilla           TTSTGPCkTCTtTAQGTSLYPSCCCTKPSDGNCTCIPIPSSWAFAKFLWEWASVRFSWL.SLLAPFVQWFAGLSPTvWLsVIWMmWYWGPNLYNILsPFIPLLPIFFCLWVYI   226
Orangutan         TTSvGPCRTCTIsApGTSLfPSCCCTKPSDGNCTCIPIPpSWAFAKFLWgWASVRFSWLn.LLvPFVQWFAGLSPTvWLsVIWMIWYWGPsLYNILsPFIPLLPIFFCLWaYI   226
Woolly monkey     TTtTGPCRTCTpivpGiSsYPSCCCTKPtDGNCTCIPIPSSWAFAKFLWdWAlaRFSWLnSLL.PFVQWFAGLSPTvWLLVIWMmWfWGPsLfsILsPFlPLLPlFFwLWaYI   228
ground squirrel   TetTvnCRqCTIsAQdTfstPyCCClKPtaGNCTCwPIPSSWAlgsyLWEWAlVRFSWL.SLLvPllQWlgGiSlTvWLLlIWMtWfWGPvLmsILpPFIPifalFFliWaYI   222
Stork             .......................................SiiFeKlqWEsvSalFSsiylLL.Psepk.slvalTfgLflIWttsssvtqvlvtLtqlatLsalFFknsa         169
Crane             .......................................StiFAKlqWEsvSalFSsisSLL.Psepk.slvalmfgLLlIWttsssvtqtlvtLtqlatLsalFykn          167
Duck              .......................................StilgKlqWasvSalFSsisSLL.Psdpk.slvalTfgLslIWMtsssatqtlvtLtqlatLsalFyks          167
Snow goose        .......................................StilgKlqWEsvSalFSsisSLL.PsdQk.slvalmfgLLlIWMtsssatqtlvtLtqlatLsalFyks          167
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Figure 2: Sequence alignment of ten published hepatitis sequences isolated from different species. The different functional regions are indicated; HS: transmembrane signal sequence; H: Helix; A: "a"-determinant; C: cytosolic loop.

Modeling of the “a”-determinant (aa102-154)

Comparison of the different sequences shows absence of the common “a”-determinant (aa102-154) in the avian Hepatitis B sequences (heron, duck, stork and snow goose) (25) as previously observed by others. The fact that the common “a”-determinant is absent in some Hepatitis B sequences, indicates that this part of the S-protein should be able to fold independent of the remaining parts of the S-protein. We decided to investigate whether a good structural model could be obtained using free modeling of only a dimer of the “a”-determinant (aa102-158) connected together via a $(GGGGS)^{10}$ linker.

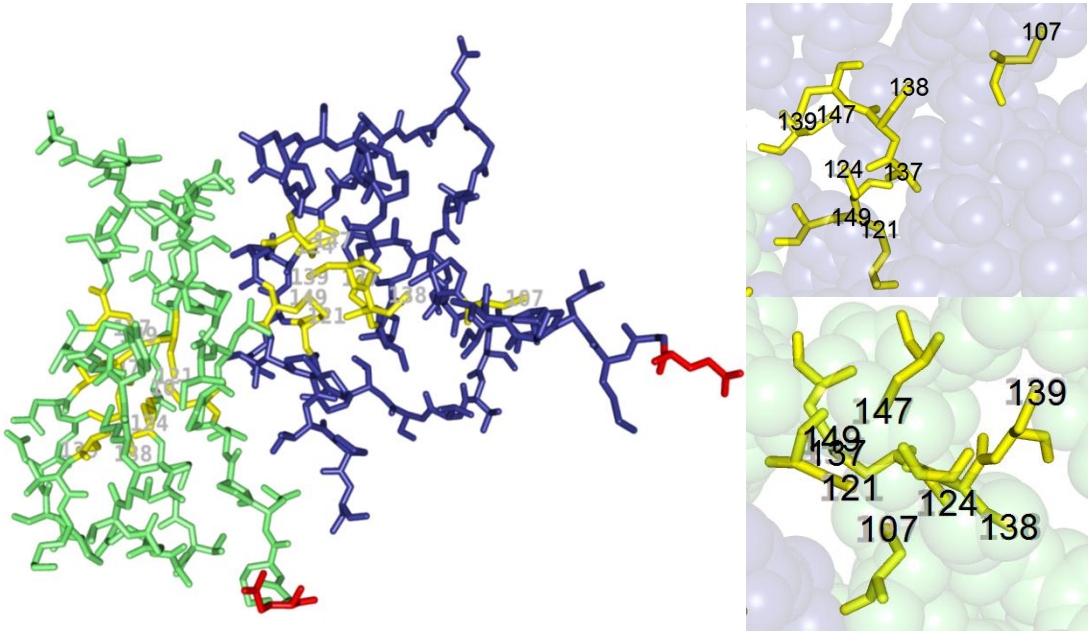

Figure 3: Dimer structure of the small S-protein aa102-158. The first amino acid of both stands is marked red. The 8 cysteines (the sulfur atom is labeled with the amino acid position in the small S-protein) present in each of the protein chains are marked yellow. The first strand is indicated in blue and the second strand in green. On the right the cysteines present in both chains are shown magnified for each chain separately (top, blue; bottom, green).

Analyzing the structures obtained we observed formation of cysteine bridges or positioning of the cysteine residues to allow formation of disulfide bridges between aa107-138; 121-149; 124-137 and 139-147 or 107-138; 121-147; 124-139 and 137-149, leaving no cysteine residues free for intermolecular bonding. The proposed two loop structure is confirmed by predicted models (9,32) although the dimer seems to support two different disulfide bond formations (figure 3). The heterogeneous nature was reported before in the light of mutational analysis and studying HBsAg mutants (9,33) confirming the predicted model with different arranged disulfide bridges in the two strands (figure 3). The accessibility of the first loop is predicted in accordance to reported antibody recognition (18,34,35), the model predicts a potential loop structure between aa107-138 with 6-8Å distance to each other, which is close enough to support disulfide bridge formation (36). The disulfide bond formation between aa121-124 was not predicted (18) in this model, but its existence lacks consensus in the scientific community (18,33). Previously we took notice of antibodies reactive against the first loop sequence (37), which could be inhibited with linear HBs peptide analogues, suggesting that the first loop is presented as a linear sequence. Also we observed increased reactivity of this linear epitope recognized by the monoclonal antibody HBs.OT17 (37) when we tested a C147S mutant, a mutant that destroys the disulfide bridge between 121 and 147 that was present in the predicted structure. The second loop structure is folded in accordance with our current knowledge; firstly both strands expose the 146N which can be glycosylated. Secondly, amino acids known involved in antibody binding within the second loop are exposed in solution. Also here we found two strands that are folded slightly differently, supporting the suggested heterogeneous nature of the “a”-determinant (9,33).

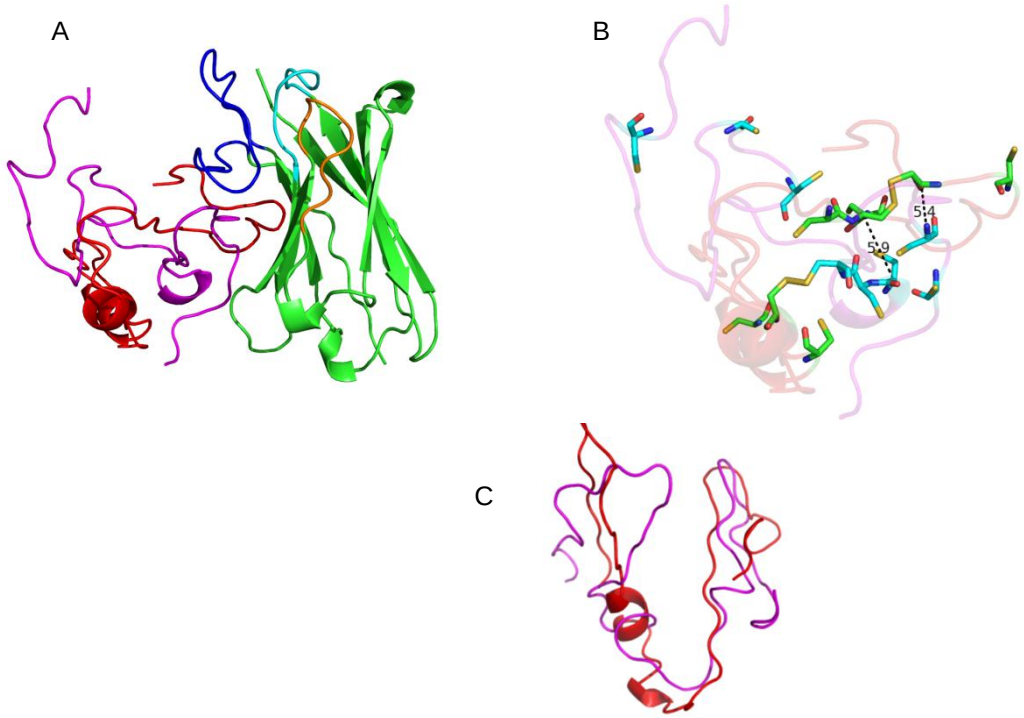

Figure 4: A: Structure of the dimer “a”-determinant with VHH S5. The complementarity determining regions (CDR’s) of the VHH are indicated in brown (CDR1), light blue (CDR2) and blue (CDR3). B: The cysteines present in the dimer “a”-determinant strands are highlighted on the right, with the distance in ångstrom indicated with the black dotted lines. The backbone of the cysteins from chain A are green and from chain B in blue. C: The two “a”-determinant strands superimposed on each other.

Building on our initial attempt to gather structures matching with the current scientific knowledge of HBsAg, we decided to dock a neutralizing VHH S5 (26, Roosmalen et al. to be published) to the dimer “a”-determinant. The VHH was used as a docking partner for the “a”-determinant assuming that the VHH would facilitate in stabilizing the structure formation during the “in silico” folding iterations. The found structure (S65035 0.41±0.14 (TM-score) C-score=-2.59 12.6±4.3Å (RMSD), figure 4) revealed a symmetrical structure of the two “a”-determinant strands and a cysteine cluster that supports inter disulfide bridging of the two “a”-determinant protein chains and intra disulfide bridging supporting loop structures between C121/124 and C137/139 and between C139 and C147/149 confirming the earlier predicted models (9,32). Intriguingly the cysteine knot visualized in this model supports compact folding of the “a”-determinant (30x40 å) with two distinct faces in a dimer structure, allowing binding of antibodies reactive with either the first (aa121-137) or second loop (139-149) structure. The importance of the cysteines for a correctly folded structure is emphasized in this model and has been shown in

experimental data by us and others (7,9,37,38). The current data shows that the “a”-determinant is on one hand a conformational structure highly sensitive for changes in any of the cysteine residues (38,39), while on the other hand can display microstructures recognized by antibodies only affected by some of the cysteine mutations (38,39). Twelve of the in total 16 cysteine residues in the cysteine knot are found within 8 Å radius (figure 4), suggesting possible disulfide bridging between any of the 12 cysteines. The central position of the three conserved cysteines (137-139) in the found structure harbors the core for the surrounding cysteines.

In the predicted model the VHH S5 is facing the dimer “a”-determinant towards the cysteine knot with interactions mainly with the second loop of the “a”-determinant and the CDR3 of the VHH. According to the model, amino acids 109-GHFDPLDLGSR-119 of VHH S5 closely interact with 144-DGNCT-148 (chain A) & 142-PSDGNC-147 (chain B) of the dimer “a”-determinant structure. Remarkably amino acids 37-VRQAPGKGPEW-47 of the VHH framework 2 are also interacting with 143-SDGNCTCIP-151 (chain B) of the “a”-determinant in such a way that framework 2 and CDR3 are embracing the dimer second loop structure of the “a”-determinant

Modeling of the c-terminus (aa155-226)

The predicted “a”-determinant structure is 30x40 Å in size, which is closely matching with the footprint of the protruding spikes observed by cryo-electron microscopy of hepatitis B virions (23). Seitz identified the footprint of the spikes of 25x45 Å that were thought to originate from 7-8 alpha helixes that pass the lipid membrane structure. It has been shown that the S-protein passes the membrane at least twice but it’s highly questionable that the protein passes the membrane again. We observed reactivity with antibodies in parts of the S-protein supposed to pass the membrane structure (21, Roosmalen et al. to be published) while others shown anti-S reactivity against dHBV and showed that these antibodies can neutralize dHBV infections (40). The fact that neutralizing antibodies excists in regions supposed to pass membranes proves visablitiy of these regions. We therefore propose as suggested by Chen (18) that the C-terminal part beyond the “a”-determinant is partially embedded in or positioned on top the lipid membrane. This peripheral position suggest that the lipids in the HBsAg particles are not organized in a typical lipid bilayer structure, but are located at the surface of the particles in a highly immobilized state as suggested by others (12,41).

The peripheral topology hypothesis was tested by submitting the C-terminal part of the S-protein (aa155-226). The found structure (figure 5) confirms the reported helical structure (25), but also showed interaction with small molecules. One of the structures revealed interaction with 1,2-dimyristoyl-sn-glycero-3-phosphocholine, a

phosphatidylcholine derivative, with the C-terminal part of the S-protein. The phosphatidylcholine derivative is a molecule highly similar to lipids found in HBsAg particles (13). Looking at the amino acids involved in the interaction we observe that W172, L173 within the C-terminus interact with the phosphocholine group, while L175 interacts with the hydrophobic tail. The high number of tryptophan residues in the C-terminal fragment suggests an interfacial orientation commonly known for peptides with tryptophan residues, which are strongly attracted to the interfacial region of lipid bilayers (42). The hypothesis is confirmed by the fact that 7 of the 9 tryptophan residues are facing the same direction as W172, shown to interacting with a lipid like molecule (figure 5).

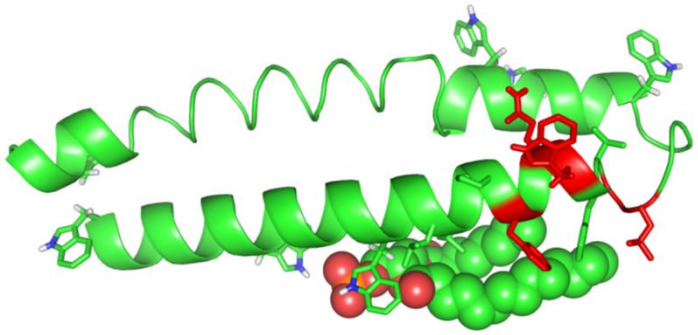

Figure 5: Predicted structure of the C-terminal part of the S-protein with the predicted position of 1,2-dimyristoyl-sn-glycero-3-phosphocholine. The nine tryptophan residues are presented as solid sticks as well as the two leucines thought to interact with the 1,2-dimyristoyl-sn-glycero-3-phosphocholine. The 4-7B epitope (21) is indicated with solid sticks and the key amino acids involved in antibody interaction are indicated in red

The proposed structure of the C-terminal part of the S-protein positions the 4-7B epitope in the turn of the protein helical structure with all four (F179, Q181, W182 and L186) key amino acids involved in the epitope recognition accessible for interaction.

Structural alignment of the model with published structures (TM-align) showed remarkable structural homology with the membrane bending protein Arfaptin 2 (TM score=0.77) (43). The protein is found positioned on top of membranes and has direct interaction with the lipid membrane via the V-shaped dimer protein structure resulting in a curved membrane. Projection of the function and topology explains the accessibility of the 170-190 region (21), but is in contradiction with previous published models that predict trans membrane positioning of the two predicted alpha helixes (4,25). Arfaptin 2 belongs to the BAR-domain family that contains a variety of members, most of which have a role in transport and endocytosis, with the BAR domain as their common feature (43). Interestingly, the function of this protein family during endocytosis/exocytosis could point to a similar function of the

C-terminus during secretion or internalization. Rearrangement of BAR proteins at specific membrane regions assists processes where bending of the membrane plays an important role, such as exocytosis (44). This hypothesis is confirmed in studies where parts of the S-protein were deleted and the authors showed that the C-terminal / bar-like part of hepatitis viruses is essential for proper secretion (15,45). During endocytosis the merger of vesicles and plasma membranes leads to formation of a fusion pore. The fusion pore is an aqueous channel connecting the vesicle lumen with the extracellular medium and is most likely a region of high curvature (44). If this function is true for HBV we speculate that HBV particles are internalized via clathrin-dependent endocytosis and transported to lysosomes, where after fusion of the S-protein the core particle can escape from the late endosome compartment (46). The exposure of the C-terminal part of the S-protein is also confirmed by direct recognition by antibodies (21), as well as by antibodies that recognize the “a”-determinant in combination with the C-terminal part (Roosmalen 2010 to be published, 18, 22). More importantly, we showed neutralization in the HDV infection model with the VHH S5 antibody confirming the hypothesis that the C-terminus is important for infectivity (Roosmalen 2010 to be published).

In conclusion, we present for the first time an '*ab initio*' structural model of the C-terminal fragment of the S-protein. We present the model as a dimer protein fragment that exits the lipid membrane placing the “a”-determinant on top of the membrane structure and placing the remaining C-terminal fragment peripheral stabilized via tryptophan and leucine residues. We propose that the HBV infection is initiated by binding of the PreS1 protein to a to-be-identified receptor (47), followed by non-specific membrane fusion mediated by the C-terminus of the S-protein. Leading to a conformational change of the I-BAR structure of the C-terminus to a F-BAR structure.

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Summary and conclusions

Diagnosis of the hepatitis B Virus (HBV) is important for treatment and prevention of further spread of the virus. Currently, detection of hepatitis B virus surface antigen (HBsAg) is the method of choice for the screening and initial diagnosis of HBV. Genetic diversity and discovery of HBV variants with mutations in the immunological dominant region of HBsAg caused none reactivity in some diagnostic assays. The lack of HBsAg detection as a result of these mutations stressed the importance of correct selection of anti-HBs antibodies. The broad detection spectrum of the monoclonal antibodies used for capture and detection of HBsAg, is therefore important for the state of the art HBsAg screening assay. The first chapter in my thesis describes the characterization of monoclonal antibodies candidates. We found most of the tested antibodies (71%), immunized and selected with wild type HBsAg, were poorly or not reactive with the most frequently found HBsAg mutant G145R. The observation that most antibodies are not reactive with the G145R HBsAg mutant correlates well with the fact that this is a dominantly found mutant after liver transplantation using Hepatitis B immunoglobulin (HBIG) only. Here the HBIg, mainly composed of antibodies sensitive for the G145R mutation, neutralizes wild type virus and selects the G145R HBsAg escape mutant. Every single mAb displayed a specific binding pattern, illustrating the abundance of the antigenic properties of the “a” determinant. Despite the great diversity, we could group the antibodies based on the global immunological reactivity of the antibodies with the HBsAg mutants and lead to classification of the antibodies into 8 groups. We concluded that the “a”-determinant is a complex conformational structure that is affected by single amino acid substitutions such as G145A/R. We noted for example that changes of amino acids in the second loop of the model presented by Stirk et al, affects the confirmation of the first loop structure making it more linear. The linear nature was suspected as we observed increased reactivity of the antibody HBs.OT17. The antibody is reactive with peptides (aa120-130) corresponding with the first loop of the “a”-determinant. A possible explanation could be rearrangement of the cysteine residues present in the “a”-determinant as a result of amino acid changes in the predicted second loop structure.

In our quest for monoclonal antibodies reactive with all HBsAg variants and mutants, we discovered a human monoclonal antibody (4-7B) reactive with all the recombinant expressed HBsAg mutants. Pepscan analysis of the monoclonal 4-7B with peptides covering the small s-protein revealed reactivity of the antibody with peptides containing the sequence between amino acids 178 - 186 (PFVQWFVGL). Single amino acid substitutions at positions 122(R/K), 134(Y/F), 145(G/R), 148(T/A) and 160(K/R) did not affect the binding of 4-7B, while replacements in the region 178-186 hampered the 4-7B HBsAg recognition. Later in our group (thanks to Piet Hiemstra) we showed reactivity of the monoclonal with all nine HBsAg

subtypes when the antibody is coated on plates, while in solution reduced reactivity is observed with HBsAg of subtype adw4. In related experiments we also confirmed reactivity with the PFVQWFVGL sequence showing a dose dependent inhibition of wild type HBsAg with a peptide containing the PFVQWFVGL sequence (IC50=0.056 µg), proving definite evidence of solution orientation of the third and fourth transmembrane region of the c-terminus within the small s-protein.

Knowledge of antibody reactivity with HBsAg variants and mutants combined with the immunological reactivity of HBsAg expressed in HepG2 cells, allowed us to design a new HBsAg screening assay, the Hepanostika HBsAg Ultra. The selection of HBs.OT40 (representing loop 2 reactive antibodies) and HBs.OT48 (representing loop 1 antibodies) combined with 4 7B was able to detect all tested HBsAg mutants and would theoretically be able to detect all HBsAg variants/mutant due to the use of three different separated binding epitopes. Early detection and specific detection of HBsAg is eminent for an accurate diagnostic assay and is shown with the new HBsAg Ultra assay. The high sensitivity of the immunoassay was achieved by using high affinity antibodies for the capture of HBsAg in solution and the use of affinity purified polyclonal anti-HBs labeled with HRP to form a specific antigen antibody complex. The high specificity of the test was achieved by counteracting interferences by addition of HRP and IgG scavengers. We showed that the Hepanostika HBsAg Ultra could detect as low as 0.08ng/ml HBsAg, while maintaining a specificity of 99.95%. Compared with other high performing HBsAg screening assays we could detect of all tested HBsAg mutants, while the other HBsAg assays clearly revealed detection problems with either, loop1, loop2 or a combination of loop1 and 2 mutants.

The improved HBsAg screening assay permits better and earlier diagnosis of HBV infections, leaving the diagnosed HBV patient for treatment. Today the main motivation for intervening in an on-going HBV infection is in case the liver is compromised. Depending on the status of the patient, antiviral therapy is started (IFN, lamivudine, and adefovir/ entecavir) or in worse case when the liver stops to function, a liver transplantation is the final choice. Transplantation of the liver is preceded by antiviral therapy in combination with HBIg additions. In a quest for an alternative source for HBig we isolated and selected single-domain antibodies (VHHs) that recognize the major small s-protein of HBV. Testing of five VHH in an in-vitro neutralization experiment identified one VHH that could neutralize the virus comparable with good neutralizing anti-HBs reference antibodies. The VHH S5 was found broad neutralizing and able to neutralize most important HBV genotypes A-E. In an attempt to find the binding epitope of the VHH we found initially conflicting results. Pepscan analysis made us suspect that the epitope of S5 is located in the c-terminus (188-220) of the s-protein, while amino acid substitution experiments suggested a conformational dependent epitope within the common “a”-determinant. The common data suggest to our opinion mutual recognition of the

VHH of both the “a”-determinant and c-terminus of the s-protein, fixating the structure and preventing conformational changes needed during viral entry. Alternatively binding to a yet to be discovered HBV (co-) receptor is blocked by binding of the VHH to the s-protein. The involvement of the small s-protein has been extensively discussed, however until today it is unclear exactly what the role is of the s-protein during the first steps of the viral entry.

Intrigued by the ability of the VHH to neutralize HBV, we tried to model the VHH on the c-terminal part of the small s-protein (aa107-226) by ab initio modeling solely. Modeling of the dimer “a”-determinant sequence with the VHH S5 revealed a tempting structure e that fitted well with the assumptions in the literature. The model shows for the first time a dimeric structure with most cysteine residues facing each other and allows formation of inter chain disulphide bridges, this in contrast to other published models that did not show inter chain disulphide bridges. The c-terminal hydrophobic region (155-226) was analysed separately and showed structural homology with Arfaptin 2, a BAR domain protein. The homologous structure of the c-terminal part of the s-protein might indicate to the importance of this sequence with respect to the function of BAR domain proteins, in this case giving curvature during the aggregating process of the s-proteins assembling on the ER. Combining the modelling data of this study with cryo-electron microscopic studies of hepatitis B virus particles, suggests that the protruding spikes are the dimers of the “a”-determinant structure, while the empty region between the 3- and 5-fold axis is filled with peripheral stabilized c-terminal fragments.

Samenvatting en conclusies

Diagnose van het hepatitis B virus (HBV) is van belang voor de behandeling en preventie van verdere verspreiding van het virus. Momenteel is de meest gebruikte methode voor de opsporing van HBV, het testen op de aanwezigheid van het hepatitis B virus oppervlakte antigeen (HBsAg). Genetische variatie en de ontdekking van HBV varianten met mutaties in de immunologische dominante regio van HBsAg resulteerde in het niet kunnen detecteren van HBsAg in een aantal diagnostische assays. Het niet kunnen detecteren van HBsAg ten gevolge van deze mutaties benadrukt het belang van correcte selectie van anti-HBs antilichamen. Gebruik van monoklonale antilichamen met een breed detectie spectrum voor HBsAg, is daarom belangrijk bij het tot stand komen van een “state of the art” HBsAg screening test. Het eerste hoofdstuk van mijn proefschrift beschrijft de karakterisatie van kandidaat monoklonale antistoffen. We vonden dat de meeste van de geteste antilichamen (71%), geïmmuniseerd en geselecteerd met wild type HBsAg, slecht of niet reactief waren met de meest voorkomende HBsAg mutant G145R. De observatie dat de meeste antilichamen niet reactief zijn met de G145R HBsAg mutant correleert goed met het feit dat dit een dominant gevonden mutant is na het uitvoeren van een levertransplantatie met alleen polyklonaal hepatitis B immunoglobuline (HBIG), welke het aanwezige virus moet neutraliseren ter voorkoming van een hernieuwde virale infectie. Hier neutraliseert HBIg, hoofdzakelijk samengesteld uit antilichamen gevoelig voor de G145R mutatie, het wild type virus en selecteert de G145R HBsAg mutant. Elke monoklonaal heeft een specifiek bindingspatroon wat aangeeft dat er een grote diversiteit van antigene determinanten is. Ondanks de grote diversiteit konden we toch groepen maken op basis van de globale immunologische reactiviteit van de antilichamen met de HBsAg mutanten, die heeft geleid tot een indeling van de antilichamen in 8 groepen. We zijn tot de conclusie gekomen dat de "a"-determinant een complexe conformationele structuur is die kan worden beïnvloed door enkele aminozuur substituties zoals de G145A / R. We merkten bijvoorbeeld op dat veranderingen van aminozuren in de tweede lus, zoals weergegeven in het model van Stirk et al., invloed heeft op de conformatie van de eerste lus structuur waardoor deze meer lineair werd. De lineaire structuur werd zichtbaar door een verhoogde reactiviteit van de monoklonale antistof HBs.OT17, ten opzichte van een verminderde reactiviteit gemeten met een niet gemuteerde HBsAg. Het antilichaam is reactief met peptiden (aa120-130) corresponderend met de eerste lus van de "a"-determinant. Een mogelijke verklaring zou kunnen zijn dat de cysteïne residuen in de "a"-determinant anders worden gerangschikt ten gevolge van aminozuurveranderingen in de tweede lus structuur.

In onze zoektocht naar monoklonale antilichamen die reactief zijn met alle HBsAg varianten en mutanten, ontdekten we een humaan monoklonaal antilichaam (4-7B) reactief met alle recombinant geëxpresseerde HBsAg

mutanten. Pepscan analyse van de monoklonaal 4-7B met peptiden fragmenten van het s-eiwit liet reactiviteit zien met peptiden die de sequentie tussen aminozuren 178 tot 186 (PFVQWFVGL) bevatte. Aminozuur veranderingen op posities 122 (R / K), 134 (Y / F), 145 (G / R), 148 (T / A) en 160 (K / R) hadden geen invloed op de binding van 4-7B, terwijl vervangingen in de regio 178-186 de reactiviteit van 4-7B verminderde. Later hebben we in onze groep (met dank aan Piet Hiemstra) laten zien dat, indien het antilichaam wordt gecoat op microtiter platen, alle negen HBsAg subtypes worden herkend. Dit terwijl in oplossing verminderde reactiviteit wordt waargenomen met HBsAg van het subtype adw4. In vervolg experimenten hebben we ook de reactiviteit met de PFVQWFVGL sequentie bevestigd en vonden we een dosis afhankelijke remming van het wild type HBsAg geïncubeerd met een peptide met de PFVQWFVGL sequentie (IC50 = 0,056 µg). Hieruit blijkt onomstotelijk dat het derde en vierde transmembraangebied van de c–terminus een oplossingsoriëntatie moet hebben.

De kennis van antilichaam reactiviteit met HBsAg varianten en mutanten, in combinatie met de immunologische reactiviteit gevonden met HBsAg uit HepG2 cellen, stelde ons in staat een nieuwe HBsAg screeningstest, de Hepanostika HBsAg Ultra te ontwerpen. De keuze van HBs.OT40 (reactief in de tweede lus) en HBs.OT48 (reactief in de eerste lus 1) gecombineerd met 4-7B resulteerde in assay ontwerp welke alle HBsAg mutanten kan opsporen. In theorie zou deze in staat zijn om alle HBsAg varianten / mutanten te detecteren door het gebruik van drie verschillende gescheiden epitoop gebieden. Vroege en specifieke detectie van HBsAg is belangrijk voor een goede diagnostische test en wordt aangetoond met de nieuwe Ultra HBsAg test. De hoge gevoeligheid van de immunoassay werd bereikt door gebruik te maken van antilichamen met hoge affiniteit voor het invangen van HBsAg uit oplossing en het gebruik van affiniteit gezuiverd polyklonaal anti-HBs gelabeld aan HRP voor het vormen van een specifiek antigeen antistof complex. De hoge specificiteit van de test werd gerealiseerd door het tegengaan van mogelijke verstoringen veroorzaakt door het niet specifiek binden van het reporter enzym HRP of het affiniteit gezuiverd polyklonaal anti-HBs-HRP. De mogelijke verstoringen werden tegen gegaan door toevoeging van geïnactiveerd HRP en IgG “scavangers”. In een uitgebreide studie waarin de Hepanostika HBsAg Ultra werd vergeleken met andere diagnostica vonden we een gevoeligheid van 0.08ng/ml met behoud van een specificiteit van 99,95%. In vergelijking met andere “state of the art” HBsAg screeningstesten konden we alle geteste HBsAg mutanten detecteren, in tegenstelling tot de andere HBsAg testen, waar duidelijk naar voren kwam dat detectie van lus 1, lus 2 of een combinatie van lus 1 en 2 mutanten problematisch was.

De verbeterde HBsAg screeningstest maakt een betere en vroegere diagnose van HBV-infecties mogelijk, waardoor de diagnose van een HBV infectie eerder kan worden gesteld en de patiënt eerder voor een behandeling in aanmerking

komt. Vandaag de dag is de belangrijkste motivatie voor het ingrijpen bij een HBV infectie het moment waarbij vast is komen te staan dat de lever is aangetast. Afhankelijk van de status van de patiënt wordt de behandeling met antivirale therapie (IFN, lamivudine en adefovir / entecavir) gestart of in het ergste geval als de leverfunctie stopt, wordt als laatst mogelijke optie de lever getransplanteerd. Transplantatie van de met HBV geïnfecteerde lever wordt voorafgegaan door antivirale therapie gecombineerd met HBIg. In een zoektocht naar een alternatieve bron voor HBIg isoleerde en selecteerde we enkel-domein antilichamen (VHHs) die het s-eiwit van HBV herkennen. Het testen van vijf VHH's in een in-vitro neutralisatie experiment, identificeerde één VHH die het virus goed neutraliseerde, vergelijkbaar met goed neutraliserende anti-HBs antistoffen. De VHH was in staat om de belangrijkste HBV genotypen A-E te neutraliseren. In een poging om de epitoop van VHH S5 te vinden, vonden we in eerste instantie tegenstrijdige resultaten. De pepscan analyse deed ons vermoeden dat de epitoop van S5 is gelegen in de c-terminus (188-220) van het s-eiwit, terwijl de aminozuur substitutie experimenten veronderstelde dat het gaat om een conformationele epitoop in de "a"-determinant. De gemeenschappelijke data suggereert naar onze mening dat de VHH zowel de "a"-determinant als de c-terminus van het s-eiwit herkent en de structuur fixeert om te voorkomen dat conformationele veranderingen plaats vinden. Een andere mogelijkheid zou kunnen zijn dat er een nog niet ontdekte HBV (co-) receptor geblokkeerd wordt door binding van de VHH aan het s-eiwit. De betrokkenheid van het s-eiwit is al uitvoerig besproken, maar tot vandaag is onduidelijk wat zijn rol precies is tijdens de eerste stappen bij het binnendringen van het virus.

Geïntrigeerd door het vermogen van de VHH om HBV te neutraliseren, hebben we geprobeerd om de VHH op het c-terminale deel van het s-eiwit (aa107-226) te modeleren gebruikmakend van "*ab-initio*" modellering. Modellering van de dimeer "a"-determinant sequentie met VHH S5 resulteerde in een verleidelijke structuur die goed past bij de veronderstellingen in de literatuur. Het model toont voor het eerst een dimeer structuur waarbij de meeste cysteïne residuen naar elkaar toe gericht zitten, om zo de vorming van interketen disulfide bruggen mogelijk te maken. Dit in tegenstelling tot andere publicaties waarbij de cysteïne residuen te ver van elkaar verwijderd zijn om dit toe te laten. Het c-terminale hydrofobe gebied (155-226) werd afzonderlijk geanalyseerd en toonde structurele homologie met Arfaptin 2, een BAR domein eiwit. De homologe structuur van het c-terminale deel van het s-eiwit zou kunnen wijzen op het belang van deze sequentie met betrekking tot de functie van BAR domein eiwitten. In dit geval het geven van kromming tijdens het aggregatie proces op het ER. Het combineren van de modelleringsgegevens van deze studie met cryo-elektronen microscopische studies met HBV partikels, suggereert dat de uitstekende spikes de dimeren van de "a"-determinant structuur zijn. Terwijl het lege gebied tussen de drie- en vijfvoudige as is gevuld met perifeer gestabiliseerd c-terminaal s-eiwit.

Curriculum Vitae

Mark van Roosmalen werd geboren op 13 januari 1970 te Geldrop. In 1988 behaalde hij het eindexamen HAVO op de Bracbant HAVO in Boxtel. In hetzelfde jaar begon hij met de hogere laboratorium opleiding aan de Fontys hogeschool Eindhoven. Hij koos voor de studie richting algemene microbiologie en behaalde het HLO diploma in januari 1993. In dezelfde maand trad hij in dienst bij Organon Technika in Boxtel als research analist onder leiding van Dr. Wilma Paulij, waar hij heeft bijgedragen aan de ontwikkeling van een anti-HBs test. De periode tussen 1994 en 1998 was hij werkzaam op de afdeling Biomaterial Research Unit, waar de basis werd gelegd voor het promotie onderzoek. Nadat Dr. Wilma Paulij Organon Technika verliet, kreeg de auteur de gezamenlijke verantwoordelijkheid voor hepatitis B exploratie groep gedurende de periode van 1998 tot 2001 onder leiding van Dr. Jan-Albert Hellings. Later, in de periode 2003 tot 2006 werd de auteur groepsleider immunoassay research onder leiding van Dr. Jacques de Jong en startte hij met een master opleiding aan De Montfort University te Leicester. In 2007 behaalde hij zijn Master of Science diploma in de richting Virologie. Mei 2009 begon hij als manager binnen Future Diagnostics, waar hij verantwoordelijk was voor de ontwikkeling van diagnostische assays. Naast zijn werkzaamheden als manager, begon hij aan de laatste fase van zijn promotie onderzoek bij Prof. Theo Verrips en Prof Hans de Haard binnen de faculteit bètawetenschappen aan de Universiteit van Utrecht, waar hij het proefschrift met het beschreven onderzoek heeft gecompleteerd. De auteur is momenteel nog steeds werkzaam bij Future Diagnostics, verantwoordelijk als manager voor de ontwikkeling van point of care testen op het MagnoTech platform van Philips.

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