Development of monoclonal antibodies to target the large surface protein of Hepatitis B virus and their use in therapeutic and diagnostic applications

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Abstract:

Over 250 million people are living with chronic infection caused by the Hepatitis B virus (HBV). HBV has 3 surface proteins, namely small (SHBs), medium (MHBs), and large (LHBs), and they play different roles in the virus life cycle. The approved hepatitis B vaccine only contains the SHBs protein and much studies have focused on characterizing the functional domains in SHBs. Although the LHBs protein is less studied, recent studies have shown that it plays important roles in mediating viral entry, replication and assembly. Over the years, there have been major advancements in monoclonal antibody (mAb) discovery tools and multiple mAbs have been developed to specifically target the preS1 domain in LHBs. We summarize the HBV infection systems and antibody discovery strategies that have been utilized by various research groups to assess the potential use of anti-preS1 mAbs as therapeutic antibodies against HBV or in the development of new diagnostic assays.

Introduction

Hepatitis B virus (HBV) is a partially double-stranded DNA virus that belongs to the family of Hepadnaviridae and has a strict tropism for human liver. Although epidemiological studies have shown that most HBV patients recover, approximately 5-10% of the cases progress to chronic hepatitis B (CHB) virus infection, with an elevated risk of developing liver cirrhosis and hepatocellular carcinoma (HCC)[1] . With 1.5 million new infections every year, the World Health Organization (WHO) estimated that over 250 million people were living with CHB in 2019, from whom an approximate of 820,000 deaths were reported, primarily due to liver cirrhosis and HCC (https://www.who.int/news-room/fact-sheets/detail/hepatitis-b. Accessed on November 10, 2022). While an effective vaccine is currently widely available, the development of a cure for CHB remains challenging, predominantly due to the persistence of a viral nuclear covalently closed circular DNA (cccDNA) that is not targeted by existing therapeutic interventions[2-4]. Such persistence of HBV infection may also be fueled by host's immune responses to the genetically diverse intra-host HBV populations that can evade the host- and therapeutic-induced selection pressures[5].

To date, HBV is classified into four subtypes (adw, adr, ayw, and ayr) and nine confirmed genotypes (genotypes A to I), and one putative genotype J, all of which further diverged into numerous subtypes with varying modes of transmission, pathogenicity, and geographical distribution[5-7]. For instance, genotypes A and D are more commonly reported in Europe, while B and C are the dominating genotypes found in the Asia[8]. Importantly, a meta-analysis assessing the risk of HCC in patients with different HBV genotype revealed significantly more HCC were reported among genotype C-infected patients than those with genotypes A or D[9].

Structurally, the HBV virion is approximately 42 nanometer (nm) in diameter and comprises the nucleocapsid, viral genome, and envelope proteins. The HBV genome **(Figure**

1A) has a relaxed genome size of 3.2 kilobases (kb), containing four open reading frames (ORFs), namely the pre-core/core, polymerase, pre-S/S, and X[10]. Nucleocapsids, which are made of core antigen (HBcAg), act as a capsule that houses the HBV genome and polymerase. There are three HBV envelope proteins which are collectively known as HBV surface (HBs) proteins, namely the small, medium, and large HBV surface proteins, and are abbreviated as SHBs, MHBs and LHBs, respectively **(Figure 1B)**[11]. The MHBs and LHBs envelope proteins are the extended forms of SHBs protein (227 amino acids, aa) at the N-terminus, with the MHBs protein having a pre-S2 (55 aa) domain, while the LHBs protein has both the pre-S1 (108 to 119 aa, depending on genotypes) and pre-S2 domains. These proteins are present on virions as well as on subviral particles (SVPs), which are particles without a viral genome. While SHBs, the most abundant surface protein, plays a vital role in the production of HBV virions and SVPs[12], the precise function of MHBs remains elusive, although some studies show that MHBs may play a role in the secretion of virions[12, 13]. On the other hand, LHBs, which is the largest HBs, plays a critical role during the entry of HBV into the host cell. It was reported by Bruss et al. that function of LHBs includes facilitating viral invasion by acting as the receptor protein for viral invasion as well as viral assembly by acting as the matrix protein[14]. Furthermore, the structural changes of LHBs during virion maturation also suggest its potential role in viral release and HBV infectivity[14]. Importantly, studies have unravelled several immunogenic epitopes within the pre-S1 region of LHBs **(Figure 1C)** and such observation will be discussed in greater detail in the later sections.

Although antiviral therapies and vaccine are available, HBV remains a global threat to public health. Currently, the most widely available vaccine for HBV contains only SHBs and is effective in preventing HBV infection in most populations[15]. Much studies have focused on the properties of SHBs including the 'a' determinant part, which is considered as a major neutralizing epitope[16]. To improve vaccine efficacy, third generation of vaccines that contain

both pre-S and S epitopes have been tested in various clinical trials, and yielded promising observation[15]. For instance, the new generation of vaccine has been shown to improve the vaccination outcomes for certain groups of people who do not response well to the conventional vaccine. Such observation highlights the importance and the potential of LHBs, particularly pre-S1/pre-S2 domains, in stimulating protective immunity against HBV infection[15, 17]. In this review, we will summarize recent studies on the function of LHBs and highlight how monoclonal antibodies (mAbs) targeting the pre-S1 domain, which is unique to LHBs, are being used in research to develop new therapies and diagnostic assays.

The functions of LHBs in viral entry, replication, and assembly

LHBs has multiple functions and has been shown to be involved in viral entry, replication, and assembly of the virion. The HBV life cycle starts with a low-affinity interaction between HBV and heparan sulfate proteoglycans (HSPGs) present on hepatocytes[18]. The antigenic loop (also known as 'a' determinant region) of HBs is responsible for this interaction. Subsequently, a high affinity interaction occurs between the LHBs protein and the sodium taurocholate cotransporting peptide (NTCP) receptor present on liver cells[19]. This vital viral-host interaction is dependent on the residues 12 to 59 (this is based on the numbering system for the Genotype A, P31873 while the corresponding numbering system in the original publication refers to residues 1 to 48) of the pre-S1 domain, which is myristoylated at its N terminus, in LHBs[20, 21]. After the attachment step, endocytosis of virion occurs, but the exact next step for viral uncoating is not fully defined. However, recent work by Perez-vargas and co-workers identified and characterized a potential fusion peptide at residues 60 to 78 (this is based on the numbering system for the Genotype A, P31873 while the corresponding numbering system in the original publication refers to residues 48 to 60) in the pre-S1 domain of LHBs and suggests that a membrane fusion mechanism could be triggered by host factor ERp57, a protein disulfide that promotes HBV entry and infectivity[22].

The LHBs protein has two membrane topologies with distinctive roles in the viral life cycle. In one topology, the N-terminal part of LHBs containing pre-S1 and pre-S2 is present on the cytosolic side, while in the other case, N-terminal part is on the luminal side of the ER[14, 23]. During infection, the pre-S1 part is exposed on the outer side of the virion and supports the interaction with the NTCP receptor on hepatocytes[24]. While in the later stage of infection, the pre-S1 part of the LHBs protein is present in the cytosolic side and is needed for viral assembly[25]. For this form of LHBs, there is the cytosolic anchorage determinant motif in pre-S1 residues 82 to 106 (this is based on the numbering system for the Genotype A, P31873 while the corresponding numbering system in the original publication refers to residues 70 to 94), which binds to Heat shock cognate protein 70 (Hsc70), and a matrix domain which is from ~residue 98 (this is based on the numbering system for the Genotype A, P31873 while the corresponding numbering system in the original publication refers to residue 86) to the end of pre-S1 and extending into pre-S2 (see Figure 1C and recent review by Seitz[26]). The matrix domain has been shown to interact with the HBV capsid protein to regulate virion formation. Importantly, LHBs interacts with ɣ2-adaptin, which is a putative member of the clathrin adaptor proteins responsible for protein sorting and trafficking in the cytoplasm, suggesting that this interaction may be important for viral assembly[27]. It has been reported by Summers et al. that LHBs are responsible for initiation of replication of cccDNA minichromosomes leading to their increase in the copy number[28]. These correlate well to HBV DNA in turn highlighting the replication status of HBV in patients, suggesting LHBs' role as a marker of viral infection and replication. The pre-S1 on the cytosolic side also controls the viral replication by regulating the amplification of supercoiled viral genome[28]. However, LHBs may also negatively regulate HBV transcription as it has been shown to interact with the Translocase of Inner Mitochondrial Membrane 29 (TIMM29), which is an antiviral host factor localized to the inner mitochondrial membrane [29].

3. Chronical events in the development of neutralizing mAbs targeting the pre-S1 domain in LHBs

Two main groups of neutralizing mAbs have been produced to prevent HBV infection. The first group of neutralizing mAbs is against 'a' determinant which is common in all 3 HBs proteins. One such example is the mAb GC1102 which is being evaluated in a phase II clinical trial[30]. There are two mechanisms of action for GC1102: first is by forming immune complex with the circulating virions or SVPs, and the second is by preventing the re-entry of the virus by interacting with SHBs[31]. Other promising therapeutic mAbs belonging to this group have also been recently discussed in a review paper[32]. The second class of neutralizing mAbs targets the pre-S1 domain of the LHBs and selected anti-pre-S1 neutralizing mAbs generated by different laboratories and their neutralizing epitopes are listed chronologically in Table 1.

For these selected mAbs, this section gives a summary of the immunogen designs, antibody library or technology, epitope mapping and neutralizing assays used in each study:

***BX-182*:** Hybridoma technology was used to isolate mAbs from mice immunized with 22nm spherical particles containing different HBs proteins which were prepared using an ultracentrifugation process[33]. One mAb named as BX-182 was found to bind strongly to *ad*-related subtypes and their corresponding A, B, C, F, H genotypes of LHBs but showed no significant binding to *ay*-related ones (or in genotypes D, E, G). The *in vivo* neutralizing activity of BX-182 was then demonstrated by using chimpanzee infected with an *adw* subtype of HBV. Epitope mapping via a random peptide phage library revealed that BX-182 bound to residues 17 to 21 of the pre-S1 domain in LHBs[34].

***MA18/7*:** HBV particles, which were obtained from the plasma of HBV carriers using sucrose gradient, were used for mouse immunization and the hybridoma clone for mAb MA18/7 was obtained using standard fusion of mouse B cells with a myeloma cell line[35]. By using overlapping peptides to determine the binding epitope of MA18/7, the minimal region was found to be DPAF (residues 31 to 34)[36]. Consistent with the conservation of this epitope, MA18/7 was found to bind to peptides from 3 different subtypes, namely *ayw, adw*, and *adr*[37]. Initially, the ability of MA18/7 to block the attachment of pre-S1-containing infectious virus particles and SVPs (filamentous) to the human liver cell membrane was observed[38]. Subsequently, the neutralizing activity of mAb MA18/7 was confirmed by its ability to block the entry and replication of HBV in Tupaia hepatocytes[39].

***KR127 and KR359*:** In this study, the N-terminal part of pre-S1 (residues 1 to 56), which contains the NTCP-binding domain, were expressed as a GST-fusion protein in *E. coli* and the GST-tag was removed by thrombin. Hybridoma technology was then used to isolate mAbs KR127 and KR359 from mice immunized with the pre-S1 fragment conjugated with keyhole limpet hemocyanin[40]. By creating a series of GST-fusion proteins containing different residues of pre-S1, the binding epitopes of mAbs KR359 and KR127 were mapped to residues 19 to 26 and 37 to 45, respectively[41]. Subsequently, the neutralizing activities of these two mAbs were confirmed by their abilities to block the infection of primary human hepatocytes with two subtypes of HBV, namely one of *adr*-subtype and another of *ayw*-subtype.

Subsequently, Hong HJ et al. developed humanized version (HzKR127) of KR127[42] and Wi, J., et al. developed humanized version (HzKR359-1) of KR359 by CDR grafting[43]. Taking the study forward, Kim JH et al, improved HzKR127 by further humanization and affinity

maturation to generate HzKR127-3.2[44]. They provided evidence that the affinities of these humanized antibodies are higher than that of their original murine counterparts. Wi, J., et al. also characterized the combined binding capacities of HzKR359-1 and HzKR127- 3.2 and observed that combination of these two humanized antibodies can bind to the preS1 region of most HBV genotypes. The result indicates that the combination of these humanized mAbs combinations may broadly neutralize HBV infection.

***2H5-A14*:** A panel of fully human mAbs binding to the N-terminal of pre-S1 were selected from a non-immune single-chain fragment of variable domain (scFv) phage display antibody library by using two synthetic peptides named as m47b and NC36b for panning separately[45]. While m47b is an N-terminal myristoylated peptide corresponding to residues 2 to 48 of LHBs, NC36b is not modified and corresponding to residues 4 to 36 of LHBs. By using HBV infection of HepG2-hNTCP cells, 6 neutralizing mAbs were identified and out of them, mAb 2H5 was found to have the most potent activity against HBV and it also neutralized hepatitis D virus. Crystallographic studies mapped the binding epitope of mAb 2H5 to residues 30 to 38 (this is based on the numbering system for the Genotype A, P31873 while the numbering system in the original publication refers to residues 19 to 27) and antibody engineering via variable heavy chain shuffling was used to increase binding affinity[45]. An improved mAb 2H5-A14 was subsequently evaluated for its ability to protect liver-humanized mice from HBV infection and showed good prophylactic and therapeutic efficacies in a Fc-dependent manner. Importantly, mAb 2H5-A14 showed similar neutralizing activities for HBV of genotypes B, C, and D although it showed limited binding to the rarer genotypes F and H.

***1A8*:** As an extension of the above study on mAbs KR359 and KR127, the same group purified bacterially expressed glutathione S transferase-tagged (GST) fusion protein containing the whole pre-S1 domain as well as synthesized a peptide corresponding to residues 20 to 32 of pre-S1[46]. These immunogens were used sequentially to pan a human synthetic fragment antigen-binding region (Fab) library in the following order: GST-pre-S1 (residues 1 to 119), GST-pre-S1 (residues 1 to 56), peptide corresponding to residues 20 to 32 of pre-S1 and untagged pre-S1 (residues 1 to 119). The 1A8 phage Fab showed the highest binding activity and was converted into human IgG1 mAb. Recombinant pre-S1 proteins of different genotypes were produced using mammalian cells and purified for Enzyme-linked immunosorbent assay (ELISA) and biolayer interferometry-based affinity measurements. The mAb 1A8 bound strongly to recombinant pre-S1 protein of HBV genotype A to C with slightly lower binding to genotype D. However, 1A8 did not bind to pre-S1 of E, F and G. A virion-binding assay showed that mAb 1A8 was able to immunoprecipitate viral particles of both genotype C and D. It also blocked the infection of HepG2-NTCP by HBV particle of genotype D[46].

***BP-L34, L41, G82, G88 & BP-L03, L07, L13, G87*:** In this study, Yato and co-workers immunized mice with different DNA constructs containing LHBs or pre-S1 fused with different tags for four times and followed by a final intraperitoneal injection of 293T cells transfected with each plasmid[47]. By testing mouse serum for neutralizing activities with a nanoluciferase reporter virus of HBV, two groups of mice (pCAG-LHBs, and pCAG-GroEL-pre-S1) showed greater neutralizing activity and were selected for B cell sorting and cloning. 13 mAbs were obtained and overlapping peptides were used to categorize them into three groups based on the epitope that they recognize, namely Epitope I (residue position relative to the Genotype A, P31873, numbering system: residues 19 to 27), Epitope II (residue position relative to the Genotype A, P31873, numbering system: residues 34 to 46), and Epitope III

(residue position relative to the Genotype A, P31873, numbering system: residues 46 to 52). The positions based on numbering system in the original publication are residues 8 to 16 (Epitope I), 23 to 35 (Epitope II), and 35 to 41 (Epitope III), respectively[47]. BP-L34, L41, G82 and G88 bound to epitope I and they could neutralize HBV of genotypes A, B and C but not genotype D. The mAbs binding to epitope II did not have neutralizing activities while mAbs binding to epitope III (BP-L03, L07, L13, G87) only neutralized genotype C virus.

Interestingly, some of the mAbs generated by different laboratories mapped to similar neutralizing epitopes in pre-S1 domain (**Figure 1C**). For example, mAbs 1A8, KR359 and BP-L34 are binding to the sequence NPLGFFP (~residues 20 to 26 of pre-S1), suggesting that this is a highly immunogenic neutralizing epitope. As this motif is essential for binding to the cellular receptor NTCP, mAbs 1A8, KR359 and BP-L34 are expected to directly block the interaction between HBV and NTCP to prevent viral entry. The binding epitopes of mAbs MA18/7 and 2H5-A14 also overlap with each other, and this epitope is further downstream (~residues 30 to 36 of pre-S1). Since this epitope (LDPAF) is just downstream of the NTCP binding site in pre-S1, mAbs MA18/7 and 2H5-A14 may be blocking viral entry by steric hindering the interaction between HBV and NTCP.

4. Comparison of antibody discovery strategies used in anti-preS1 antibody generation

Advanced antibody screening and isolation technologies have led to the discovery of the anti-preS1 therapeutic mAbs listed above. Both non-immune and immune antibody repertoires have been successfully utilized and for the latter, the success in antibody discovery is highly dependent on the immunogen used during immunization. Shih. et al utilized full-length HB proteins purified from viral particles for immunization so that there would be antibodies binding to different epitopes in the full-length proteins [33]. In contrast, Ryu, C.J et

al [40] and Maeng, C.Y., et al [41] utilized recombinant protein by using GST tagged HBV protein pre-S1 N-terminal fragment expressed and purified in *E.coli*, . In this way, they have the advantage of generating mAbs that are specific to epitopes in the preS1. Recombinant protein or synthetic peptide based immunization, while being generally successful, cannot be used for antigens with complex structures or high hydrophobicity as these pose challenges for large-scale protein purification. In this regard, Yato, K., et al [47] used an alternative method, i.e. DNA immunization with constructs containing LHBs for direct antigen production *in vivo*. The DNA immunization strategy provides an improvement over the protein-based antigens, due to production of immunogens *in vivo*, thus eliciting T-cell immune responses through endogenous antigen processing and presentation pathways. Another immunogen synthesis method was used by Heermann, K.H., et al [35] to generate MA18/7. They utilized HBV virus-like particles (VLPs) as immunogens, which have recently emerged as potent immunogens which retains the structural integrity and stability of the membrane proteins.

Albeit using different immunogen formats, most of the mAbs listed in Table 1 were generated following the classic hybridoma technology for generation of the monoclonal antibodies via immortalization and isolation of mouse B cells. Although this technology has been historically reported to generate potent neutralizing mAbs, these murine antibodies will elicit immunogenic responses in humans and are not suitable for therapeutic applications. Humanization of these mAbs is possible but can sometimes lead to decrease in binding and neutralization capabilities. Importantly, the advancement of phage display technique, with its capability of displaying of millions of antibody variants, has allowed fully human antibodies to be discovered for anti-PreS1. For example, Jo, G., et al [46] panned a phage displayed human synthetic fragment antigen-binding region (Fab) library against pre-S1 fusion protein as well as synthetic peptides, to generate mAb 1A8.

Compared to generation of mAbs from immune libraries, non-immune libraries provide the advantage of reducing antigen-induced biases in the repertoire, owing to their origin from natural, unimmunized, rearranged variable (V) genes. *In vitro* introduction of complete or specific degeneracy into the complementarity-determining regions (CDRs) of multiple V genes into synthetic antibody libraries bypasses the natural biases and redundancies of *in vivo* antibody repertoires and provides advantage of increased antibody diversity[48]. Improving upon the mAb discovery technology, Li, D., et al [45] utilized non-immune single-chain fragment of variable domain (scFv) phage display antibody library, followed by panning with synthetic peptides for generating fully human mAbs leading to mAb 2H5 discovery. Furthermore, they improved the 2H5 MAb by antibody engineering via variable heavy chain shuffling.

5. The expression of LHBs in patient samples and its clinical significance

Diagnostic assays for measuring total HBs (known also as HBs antigen, HBsAg) in patient's serum, like Architect HBsAg QT (Abbott Diagnostics) and Elecsys HBsAg II Quant (Roche Diagnostics), are routinely used to evaluate the effectiveness of antiviral therapy (as reviewed by Liaw [49]). However, these assays measure total HBV surface protein level and do not distinguish between SHBs, MHBs and LHBs because they are developed with antibodies binding to the S domain. The development of mAbs binding to the pre-S1 domain (including those with neutralizing activities as summarized in Table 1 as well as those that are non-neutralizing) and mAbs with binding specificity to MHBs[50] have allowed researchers to compare the serum levels of these proteins individually. For example, Ringer and co-workers used sandwich ELISA to compare the levels of SHBs, MHBs and LHBs in patients undergoing pegylated interferon (PegIFN) treatment and found that each of the HBs protein was significantly lower in responders than in nonresponders [51].

It is important to differentiate between HBAg-negative inactive HBV carriers and patients with active CHB infection. During the inactive HBV carrier phase, there is minimal replication of HBV, no inflammation and no or mild liver damage. While in CHB infection, there is an increased risk of developing end-stage liver disease and HCC [52]. As these two groups of patients require different treatments, it is crucial to discriminate between different phases of HBV infection. In another study, Pfefferkorn and co-workers have proposed the difference in the level of individual HBs can be used to distinguish the stages of HBV infection [53]. Their results suggest that the ratios of LHBs and MHBs can be more useful markers for distinguishing between chronic hepatitis B infection and inactive HBV carriers compared to the total HBV surface protein level.

Loss of total HBs level with or without seroconversion with no HBV DNA is taken as a cure for the HBV infection during antiviral treatment [54]. Pfefferkorn and co-workers also showed that declining LHBs and MHBs proportions during antiviral treatment (like nucleos(t)ide, PegIFN) were observed earlier than total HBs loss and could be a good predictor of responders [55]. Moreover, the changes in the proportion of LHBs and MHBs were not dependent on reduction in total HBs. Hence, the proportion of LHBs and MHBs can be a unique marker for predicting the cure of HBV infection.

Pre-S1 has been reported previously to be associated with HBsAg, HBeAg, and HBV DNA [56]. Hatooka et al., reported that, in addition to these three HBV markers, pre-S1 also correlated with Hepatitis B core related antigen (HBcrAg) [57]. HBcrAg acts as a potent serum marker of HBV, owing to its property of estimating the extent of HBV cccDNA, in the liver[58]. Additionally, it has been also observed that HBsAg loss during the antiviral treatment regime directly correlated with the reduction of the LHBs ratio, thus reflecting stable remission [59].

Following up on this premise, Hatooka et al., developed a diagnostic assay system based on measuring preS1 levels by ELISA [57]. They were able to show that CHB patients,

who became HBeAg-negative but had high HBV DNA levels, also showed higher preS1 levels and hence were more prone to develop CHB. Furthermore, even when patients show minimal hepatitis activity, the presence of higher pre-S1 levels is predictive of enhanced risk of hepatitis progression, hence, requiring administration of antiviral therapy.

Similarly, another home-made anti-pre-S1 mAb was successfully used in immunohistochemistry to detect the expression of LHBs in liver samples from patients with HBV-related HCC [60]. While total HBs staining was lower in tumour region compared to surrounding non-tumour tissues, the reverse was found for LHBs which was found in a significantly higher percentage of tumor cells than non-tumour cells. The same anti-pre-S1 mAb was also used to develop a sandwich ELISA for measuring LHBs in serum when compared to total HBs measured by a commercial HBsAg kit (Abbott ARCHITECT). The serum was taken from the patients before they underwent hepatectomy and their clinicopathologic indicators after surgery were recorded. It seems serum LHBs before surgery is a better marker than total HBs in predicting HCC recurrence after surgery[60].

6. Conclusion and future directions

HBV has 3 surface proteins, SHBs, MHBs and LHBs, in its virion and they play different roles in the virus life cycle and contribute differently to viral pathogenesis. The pre-S1 domain is only found in LHBs and has been shown to be crucial for mediating important steps in the virus life cycle by interacting with host proteins. By using different types of immunogens and antibody generation methods, multiple neutralizing mAbs have been generated to target the pre-S1 domain and prevent viral entry. A dominant neutralizing epitope NPLGFFP (residues ~20 to 26 of pre-S1 with numbering relative to the Genotype A, P31873) was found to be the binding site of 3 different neutralizing mAbs, which were generated by different laboratories using different technologies (Table 1). This is consistent with the role of this motif in mediating the interaction of HBV to the NTCP cellular receptor. While none of

the pre-S1-binding mAbs has entered clinical trials yet, further research may be directed to determine if they can synergize with existing 'a' determinant-binding mAbs that are already in clinical trials because they have different mechanisms of action.

The current diagnostic assays for measuring total HBs in the serum are routinely used to follow the natural history of infection as well as to evaluate the effectiveness of antiviral therapy but they do not distinguish between the 3 surface proteins of HBV. The availability of mAbs (both neutralizing and non-neutralizing) binding specifically to LHBs has led to several important studies to determine if the level LHBs in patient serum can serve as a better biomarker than total HBs. While LHBs levels seem to be able to distinguish between different groups of hepatitis B patients, more studies with bigger cohorts of patients will be needed to determine the best way to use these new assays. The detection of intrahepatic LHBs by using immunohistochemistry in patients with HBV-related HCC also showed that LHBs were abundantly expressed in tumour cells and further studies are required to determine how this may be related to disease progression.

The development of HBV neutralizing mAbs provides an additional arsenal in our quest towards achieving a functional cure for CHB [32]. Given the important role played by LHBs in mediating viral entry as well as contributing to viral replication and/or pathogenesis, the studies summarized have illustrated how mAbs targeting the pre-S1 domain in LHBs are being used in the development of novel therapies and diagnostic assays for CHB. Ongoing improvement in the methods of mAb development, like the advances in efficient screening tools, DNA based immunogenic presentation, host cell engineering and more robust selection criteria, will accelerate our understanding of the functional importance of LHBs.

References

1. Hu, H., et al., *Incidence and Influencing Factors of New Hepatitis B Infections and Spontaneous Clearance: A Large-Scale, Community-Based Study in China.* Front Med (Lausanne), 2021. **8**: p. 717667.
2. Revill, P.A., et al., *A global scientific strategy to cure hepatitis B.* The Lancet Gastroenterology & Hepatology, 2019. **4**(7): p. 545-558.
3. Alter, H., et al., *A research agenda for curing chronic hepatitis B virus infection.* Hepatology, 2018. **67**(3): p. 1127-1131.
4. Revill, P., et al., *Global strategies are required to cure and eliminate HBV infection.* Nat Rev Gastroenterol Hepatol, 2016. **13**(4): p. 239-48.
5. Revill, P.A., et al., *The evolution and clinical impact of hepatitis B virus genome diversity.* Nat Rev Gastroenterol Hepatol, 2020. **17**(10): p. 618-634.
6. Kramvis, A., *Genotypes and genetic variability of hepatitis B virus.* Intervirology, 2014. **57**(3-4): p. 141-50.
7. Tong, S. and P. Revill, *Overview of hepatitis B viral replication and genetic variability.* J Hepatol, 2016. **64**(1 Suppl): p. S4-S16.
8. Valsamakis, A., *Molecular testing in the diagnosis and management of chronic hepatitis B.* Clin Microbiol Rev, 2007. **20**(3): p. 426-39, table of contents.
9. Wong, G.L., et al., *Meta-analysis: The association of hepatitis B virus genotypes and hepatocellular carcinoma.* Aliment Pharmacol Ther, 2013. **37**(5): p. 517-26.
10. Summers, J., A. O'Connell, and I. Millman, *Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles.* Proc Natl Acad Sci U S A, 1975. **72**(11): p. 4597-601.
11. Bruss, V., *Envelopment of the hepatitis B virus nucleocapsid.* Virus Res, 2004. **106**(2): p. 199-209.
12. Bruss, V. and D. Ganem, *The role of envelope proteins in hepatitis B virus assembly.* Proc Natl Acad Sci U S A, 1991. **88**(3): p. 1059-63.
13. Fernholz, D., et al., *Infectious hepatitis B virus variant defective in pre-S2 protein expression in a chronic carrier.* Virology, 1993. **194**(1): p. 137-48.
14. Ostapchuk, P., P. Hearing, and D. Ganem, *A dramatic shift in the transmembrane topology of a viral envelope glycoprotein accompanies hepatitis B viral morphogenesis.* EMBO J, 1994. **13**(5): p. 1048-57.
15. Shouval, D., H. Roggendorf, and M. Roggendorf, *Enhanced immune response to hepatitis B vaccination through immunization with a Pre-S1/Pre-S2/S vaccine.* Med Microbiol Immunol, 2015. **204**(1): p. 57-68.
16. Locarnini, S. and S. Bowden, *Hepatitis B surface antigen quantification: not what it seems on the surface.* Hepatology, 2012. **56**(2): p. 411-4.
17. Grzegorzewska, A.E., *Hepatitis B vaccination in chronic kidney disease patients: a call for novel vaccines.* Expert Rev Vaccines, 2014. **13**(11): p. 1317-26.
18. Leistner, C.M., S. Gruen-Bernhard, and D. Glebe, *Role of glycosaminoglycans for binding and infection of hepatitis B virus.* Cell Microbiol, 2008. **10**(1): p. 122-33.
19. Yan, H., et al., *Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus.* Elife, 2012. **1**: p. e00049.
20. Ni, Y., et al., *Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes.* Gastroenterology, 2014. **146**(4): p. 1070-83.
21. Barrera, A., et al., *Mapping of the hepatitis B virus pre-S1 domain involved in receptor recognition.* J Virol, 2005. **79**(15): p. 9786-98.

22. Perez-Vargas, J., et al., *A fusion peptide in preS1 and the human protein disulfide isomerase ERp57 are involved in hepatitis B virus membrane fusion process.* Elife, 2021. **10**.
23. Bruss, V., et al., *Post-translational alterations in transmembrane topology of the hepatitis B virus large envelope protein.* EMBO J, 1994. **13**(10): p. 2273-9.
24. Le Seyec, J., et al., *Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain.* J Virol, 1999. **73**(3): p. 2052-7.
25. Bruss, V., *A short linear sequence in the pre-S domain of the large hepatitis B virus envelope protein required for virion formation.* J Virol, 1997. **71**(12): p. 9350-7.
26. Seitz, S., et al., *The Hepatitis B Virus Envelope Proteins: Molecular Gymnastics Throughout the Viral Life Cycle.* Annu Rev Virol, 2020. **7**(1): p. 263-288.
27. Hartmann-Stuhler, C. and R. Prange, *Hepatitis B virus large envelope protein interacts with gamma2-adaptin, a clathrin adaptor-related protein.* J Virol, 2001. **75**(11): p. 5343-51.
28. Summers, J., P.M. Smith, and A.L. Horwich, *Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification.* J Virol, 1990. **64**(6): p. 2819-24.
29. Muriungi, N.G. and K. Ueda, *TIMM29 interacts with hepatitis B virus preS1 to modulate the HBV life cycle.* Microbiol Immunol, 2020. **64**(12): p. 792-809.
30. Lee, H.W., et al. *A prospective, open-label, dose-escalation, single-center, phase 1 study for GC1102, a recombinant human immunoglobulin for chronic hepatitis B patients*. in *Hepatology*. 2018. WILEY 111 RIVER ST, HOBOKEN 07030-5774, NJ USA.
31. Lee, H.W., et al., *Efficacy of Lenvervimab, a Recombinant Human Immunoglobulin, in Treatment of Chronic Hepatitis B Virus Infection.* Clin Gastroenterol Hepatol, 2020. **18**(13): p. 3043-3045 e1.
32. Beretta, M. and H. Mouquet, *Advances in human monoclonal antibody therapy for HBV infection.* Curr Opin Virol, 2022. **53**: p. 101205.
33. Shih, J.W., et al., *Production of monoclonal antibodies against hepatitis B surface antigen (HBsAg) by somatic cell hybrids.* J Virol Methods, 1980. **1**(5): p. 257-73.
34. Zhang, P., et al., *Neutralization epitope responsible for the hepatitis B virus subtype-specific protection in chimpanzees.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 9214-9.
35. Heermann, K.H., et al., *Large surface proteins of hepatitis B virus containing the pre-s sequence.* J Virol, 1984. **52**(2): p. 396-402.
36. Sominskaya, I., et al., *Determination of the minimal length of preS1 epitope recognized by a monoclonal antibody which inhibits attachment of hepatitis B virus to hepatocytes.* Med Microbiol Immunol, 1992. **181**(4): p. 215-26.
37. Coursaget, P., et al., *Mapping of linear B-cell epitopes of hepatitis B surface antigen.* Res Virol, 1991. **142**(6): p. 461-7.
38. Pontisso, P., et al., *Identification of an attachment site for human liver plasma membranes on hepatitis B virus particles.* Virology, 1989. **173**(2): p. 522-30.
39. Glebe, D., et al., *Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus.* J Virol, 2003. **77**(17): p. 9511-21.
40. Ryu, C.J., et al., *Mouse monoclonal antibodies to hepatitis B virus preS1 produced after immunization with recombinant preS1 peptide.* Hybridoma, 2000. **19**(2): p. 185-9.
41. Maeng, C.Y., et al., *Fine mapping of virus-neutralizing epitopes on hepatitis B virus PreS1.* Virology, 2000. **270**(1): p. 9-16.
42. Hong, H.J., et al., *In vivo neutralization of hepatitis B virus infection by an anti-preS1 humanized antibody in chimpanzees.* Virology, 2004. **318**(1): p. 134-41.

43. Wi, J., M.S. Jeong, and H.J. Hong, *Construction and Characterization of an Anti-Hepatitis B Virus preS1 Humanized Antibody that Binds to the Essential Receptor Binding Site.* J Microbiol Biotechnol, 2017. **27**(7): p. 1336-1344.
44. Kim, J.H., et al., *Enhanced humanization and affinity maturation of neutralizing anti-hepatitis B virus preS1 antibody based on antigen-antibody complex structure.* FEBS Lett, 2015. **589**(2): p. 193-200.
45. Li, D., et al., *A potent human neutralizing antibody Fc-dependently reduces established HBV infections.* Elife, 2017. **6**.
46. Jo, G., et al., *Generation and Characterization of a Neutralizing Human Monoclonal Antibody to Hepatitis B Virus PreS1 from a Phage-Displayed Human Synthetic Fab Library.* J Microbiol Biotechnol, 2018. **28**(8): p. 1376-1383.
47. Yato, K., et al., *Identification of Two Critical Neutralizing Epitopes in the Receptor Binding Domain of Hepatitis B Virus preS1.* J Virol, 2020.
48. Hoogenboom, H.R., *Selecting and screening recombinant antibody libraries.* Nat Biotechnol, 2005. **23**(9): p. 1105-16.
49. Liaw, Y.F., *Clinical utility of hepatitis B surface antigen quantitation in patients with chronic hepatitis B: a review.* Hepatology, 2011. **53**(6): p. 2121-9.
50. Deepen, R., et al., *Assay of preS epitopes and preS1 antibody in hepatitis B virus carriers and immune persons.* Med Microbiol Immunol, 1990. **179**(1): p. 49-60.
51. Rinker, F., et al., *Quantitation of large, middle and small hepatitis B surface proteins in HBeAg-positive patients treated with peginterferon alfa-2a.* Liver Int, 2020. **40**(2): p. 324-332.
52. European Association for the Study of the Liver. Electronic address, e.e.e. and L. European Association for the Study of the, *EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection.* J Hepatol, 2017. **67**(2): p. 370-398.
53. Pfefferkorn, M., et al., *Quantification of large and middle proteins of hepatitis B virus surface antigen (HBsAg) as a novel tool for the identification of inactive HBV carriers.* Gut, 2018. **67**(11): p. 2045-2053.
54. Cornberg, M., et al., *The role of quantitative hepatitis B surface antigen revisited.* J Hepatol, 2017. **66**(2): p. 398-411.
55. Pfefferkorn, M., et al., *Composition of HBsAg is predictive of HBsAg loss during treatment in patients with HBeAg-positive chronic hepatitis B.* J Hepatol, 2021. **74**(2): p. 283-292.
56. Liu, X., et al., *Correlation between hepatitis B virus DNA levels and diagnostic tests for HBsAg, HBeAg, and PreS1-Ag in chronic hepatitis B.* Genet Mol Res, 2016. **15**(2).
57. Hatooka, H., et al., *Construction of an anti-hepatitis B virus preS1 antibody and usefulness of preS1 measurement for chronic hepatitis B patients: Anti-HBV PreS1 antibody.* J Infect, 2022. **84**(3): p. 391-399.
58. Wong, D.K., et al., *Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection.* J Clin Microbiol, 2007. **45**(12): p. 3942-7.
59. Liu, C., et al., *Prediction value of serum HBV large surface protein in different phases of HBV infection and virological response of chronic hepatitis B patients.* Clin Chim Acta, 2018. **481**: p. 12-19.
60. Tsai, H.W., et al., *The Serum Hepatitis B Virus Large Surface Protein as High-Risk Recurrence Biomarker for Hepatoma after Curative Surgery.* Int J Mol Sci, 2022. **23**(10).

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Figure legend

Figure 1. Schematic representation of (A) the linear genome structure of hepatitis B virus (HBV) that causes acute and chronic hepatitis, (B) the structure of HBV envelope proteins, consisting large, medium, and small hepatitis B surface proteins (abbreviated as LHBs, MHBs and SHBs). The LHBs (~400 amino acids, aa) contains pre-S1 (119 aa), pre-S2 (55 aa) and S domains (227 aa). On the other hand, MHBs (~282 aa) contains pre-S2 and S domains, while SHBs contains only the S domain. There are four transmembrane domains (I – IV) in the S domains of LHBs, MHBs and SHBs, and (C) the mapping of previously reported anti-pre-S1 neutralizing monoclonal antibodies. As BP-L03, L07, L13 and G87 have the same epitope, only BP-L03 is indicated. All the positions are relative to the Genotype A (P31873), where the amino acid numbering starts at the first start codon.