Evidence for Immune Selection Pressure Imposed by the Residue Specificity of Anti-HBs Antibodies in the Hepatitis B Immune Globulin

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Abstract

Background and Purpose: Hepatitis B immune globulin (HBIG) contains polyclonal antibodies derived from the pooled plasma of donors who are naturally immunized and/or vaccinated against the hepatitis B virus (HBV). The anti-HBs antibodies present in HBIG that target the common "a" determinant of the HBV surface antigen (HBsAg a.a. 121-149) contribute substantially to the long-term protection. The anti-HBs antibody titer is currently used to define the minimum potency of HBIG for passive immune prophylaxis. Although HBIG is administered as a standardized treatment regimen for preventing HBV re-infection after liver transplantation, using it also poses the undesirable risk of inducing "escape" mutations, hence, HBV re-infection can still occur in these liver transplant patients and remains a major unsolved problem. Interestingly, the residue specificity profile of these anti-HBs antibodies is also largely unknown.

Methodology and Results: In the present study, by screening phage display libraries and using ELISA immunoassays, at least three clusters of residues around the "a" determinant of the HBsAg including R122-T123, M133-Y134-P135 and I151-P152-S153, as well as individual amino acids such as G145, were identified to interact specifically with the anti-HBs antibodies in HBIG. These particular residues were traced back to key sites previously reported to be mutable in response to HBV vaccination or HBIG prophylaxis in liver transplant recipients who got re-infected. The analysis of the residue specificity profile of HBIG revealed that anti-HBs antibodies, in addition to neutralizing the virus, also apply a selective pressure that promotes site-specific mutations on the viral surface, thus allowing HBV to escape antibody recognition.

Conclusion: Based on the analysis, it appeared that the residue specificity of the anti-HBs antibodies in the HBIG was likely responsible for the selective pressure that drove the emergence of HBsAg variations in the potential "escape" mutants. Further understanding of this immunogenetic linkage may provide practical approaches for the improvement of HBIG to be a more effective biologic product.

Introduction

An estimated 257 million people worldwide are infected with hepatitis B virus (HBV). Despite vaccination and advances in therapy, chronic HBV infection still causes approximately 887,000 deaths each year, mostly from complications of liver cirrhosis and hepatocellular carcinoma. For patients with irrevocable liver failure caused by HBV infection, orthotopic liver transplantation (OLT) is the only definitive treatment option.

The advent of hepatitis B immune globulin (HBIG) has significantly improved transplant patient and allograft survival. Long-term use of HBIG, however, comes with a risk of the development of HBV mutants, which potentially could render the virus neutralization-resistant.

Antibody binding to the common region of "*a*" determinant on the HBV surface antigen (HBsAg) is essential in conferring protection. Over 90% of human antibodies following HBV vaccination are directed toward this region. Mutations around this region impede anti-HBs activities, thus facilitating evasion of HBIG-mediated neutralization. Nearly 75% of immunosuppressed patients with HBV reactivation carried virus with at least one mutation in the "*a*" determinant. A G145>R variant, for example, can escape neutralization despite vaccination or HBIG treatment. Interestingly, withdrawal of HBIG treatment resulted in reversion of these mutations back to the original HBsAg sequence most of the time.

Materials and Methods

Peptides, proteins, anti-HBs antibodies. All peptides were synthesized by Genscript. HBsAg peptides and their mutants were biotinylated. Recombinant HBsAg proteins of subtypes *ad* and *ay*, were purchased from Bio-Rad. HBIG lots were obtained from the NIH Pharmacy.

Partial purification of anti-HBs antibodies. Prior to phage display library screening, anti-HBs antibodies in HBIG lots were enriched by binding to and eluting from HBsAg (*ad* or *ay*) pre-coated on a 96-well plate with multiple washes in between binding and elution steps.

Screening of phage display libraries. Peptides were selected from random peptide phage display libraries (12-mer and C7C, New England BioLabs) after 2 to 3 rounds of screening using partially purified anti-HBs antibodies. Peptide sequences were deduced from DNA sequences of selected phage clones.

Sequence alignment. The sequences of selected peptides were aligned among themselves or with HBsAg based on homology and then grouped into clusters. The more common residues were identified as core residues of the anti-HBs antibody binding site (i.e., epitope).

ELISA. Biotin-labeled peptides were incubated in streptavidin-coated 96-well Maxisorp plates (Thermo Fisher) for 1 hr at room temperature followed by PBS washes. Serial dilutions of purified anti-HBs antibodies were then added and incubated for 1 hr at 37°C. After washing, goat-anti-human peroxidase-conjugated IgG (KPL) was added for a 30 min incubation at 37°C. A TMB-ELISA substrate solution (Thermo Fisher) was used for color development. Absorbance (optical density, O.D.) was measured at 450 nm using a SpectraMax M2e microplate reader (Molecular Devices).

Statistical analysis. An unpaired Student's t-test with a two-tailed p value ($p \leq 0.05$) was performed using GraphPad Prism 8 software. Error bars represent standard deviation or standard error of the mean (SEM).

Results and Discussion

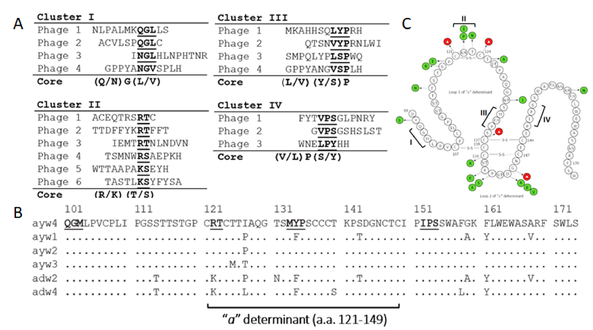

Figure 1. Identification of anti-HBs binding sites in the regions of HBsAg. (A) Four clusters of anti-HBs binding peptides were identified after screening phage display libraries (12-mer, C7C) with anti-HBs antibodies partially purified from HBIG lots. Core residues of each cluster are in bold. Letters in parentheses denote alternate residues at that position. **(B)** Alignment of HBsAg residues 101-174 of HBV serotypes. Dots represent identical residues at the respective positions compared to *ayw4*. Predicted positions of the core residues by the phage display experiments are in bold and underlined in the context of HBsAg. The "a" determinant region of HBsAg (a.a.121-149) is indicated. **(C)** Four clusters of anti-HBs binding peptides are predicted in the context of the HBsAg secondary structure.

Results and Discussion (continuation)

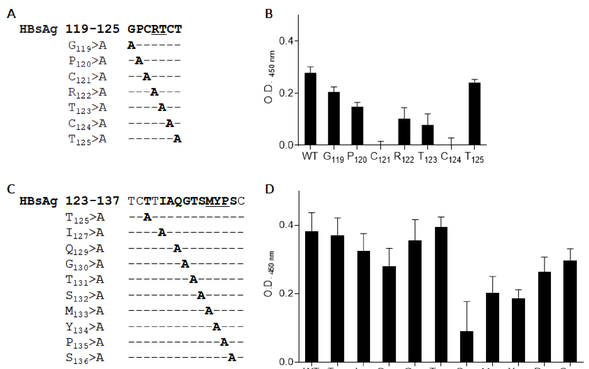

Figure 2. Alanine scanning analyses of HBsAg peptides to demonstrate residue-specific binding of HBIG. (A) Sequence of the wildtype HBsAg peptide (a.a. 119-125) and its corresponding alanine mutants. Position of alanine mutation is shown in bold. A hyphen indicates an amino acid residue identical to that of the wildtype (WT). The core residues identified by phage display experiments are underlined. **(B)** The binding of HBIG to the peptides in (A) was measured by ELISA. **(C)** Sequence of the wildtype HBsAg peptide (a.a. 123-137) and its corresponding alanine mutants. **(D)** The binding of HBIG to the peptides in (C) was measured by ELISA. X-axis indicates the residue that was substituted by alanine. Y-axis indicates the binding activity as determined by O.D. at 450 nm. Data shows the means ± SEM from triplicate readouts of one representative assay out of 3 independent assays.

A Mutations in the "*a*" determinant of HBsAg observed commonly in clinical studies

Position in HBsAg	Wildtype	Mutation	Mutant emerged	Cluster identified in this study
120	P	E-S-T	Vx-Lm-HBIG	II
123	T	N	HBIG	II & III
124	C	R-Y	mAb-HBIG	
126	T/I	A/N/S	Vx-HBIG	
129	Q	H/L	Vx	III
130	G	D/R	Lm-HBIG	
133	M	L	Vx	
134*	Y/F	N/R/S	HBIG	
141	K	E/I	Vx-mAb	
142*	P	S	Vx-mAb	
143*	S/T	L	Vx	IV
144	D	A/E	Vx-HBIG	
145*	G	R	Vx-Lm-HBIG	

The causal selection pressures are: Vx, vaccine; Lm, lamivudine; mAb, monoclonal antibody; HBIG, hepatitis B immune globulin; *The peptides containing a mutation at this position were chosen for testing in this study.

B Residue-specific Reactivity of HBIG (Clinical Significance)

HBsAg 123-137 TCTTIAQGTSMYPSC
Y_{134}>S ----------S---
Y_{134}>F ----------F---

HBsAg 139-148 CTKPSDGNCT
P_{142}>S ---S------
S_{143}>T ----T-----
G_{145}>R ------R---

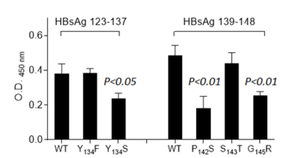

Figure 3. Potential immunogenetic linkage between the use of HBIG and emergence of HBsAg mutations. (A) Mutations in the "*a*" determinant of HBsAg commonly observed in clinical studies are listed. Causal selection pressure are: Vx, vaccine; Lm, lamivudine; mAb, monoclonal antibody; HBIG, hepatitis B immune globulin (when a mutation at a specified position of HBsAg emerges). **(B)** Sequence of the wildtype HBsAg peptides (a.a. 123-137 and 139-148) and their natural variants (Y134>F, S143>T) and clinical mutants (Y134>S, P142>S, G145>R). Mutations are shown in bold. A hyphen indicates an amino acid residue identical to that of the wildtype. The core residues identified by phage display experiments are underlined. The binding of HBIG to the peptide was measured by ELISA.

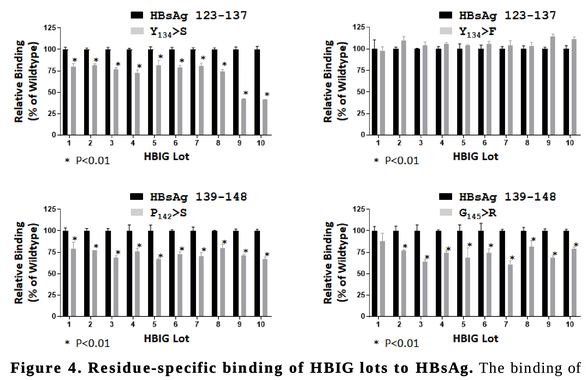

Figure 4. Residue-specific binding of HBIG lots to HBsAg. The binding of ten different HBIG lots to HBsAg wildtype peptides and peptides with clinically associated mutations was measured by ELISA. X-axis indicates the individual lots. Y-axis indicates the relative binding expressed as the % of binding by the mutant peptide compared to that of the wildtype peptide. Data shows the means ± SEM from triplicate readouts. Asterisks represent *p*-values to mark the statistical significances of observed differences.

Conclusion

HBsAg contains multiple anti-HBs binding sites that are recognized by HBIG in a residue-specific manner.

These anti-HBs binding sites were mapped to regions that encompass the "a" determinant of HBsAg, the major component of the HBV vaccine, unequivocally confirming that the anti-HBs antibodies in the HBIG product, which were collected from vaccinated plasma donors, specifically target the "a" determinant.

These anti-HBs binding sites were also located in HBsAg segments that tend to contain the mutations detected in patients undergoing HBIG therapy, suggesting that the natural variations or mutations occurring at these binding sites can modulate antibody responses and lead to immune escape of HBV.

Introducing site-specific "escape" mutations within segments of HBsAg 123-137 and 139-148 significantly diminished the binding of the anti-HBs antibodies in multiple HBIG lots.

These results suggest that while the anti-HBs antibodies in the HBIG lots act effectively to neutralize the virus to prevent HBV recurrence after liver transplantation, the sequence dynamics of HBsAg during HBIG treatment may serve as an important countermeasure for the HBV in order to escape from anti-HBs-mediated neutralization by allowing specific mutations to emerge around anti-HBs binding sites.

By providing an explanation for why liver transplant patients become re-infected despite receiving HBIG prophylaxis, our studies could contribute to the improvement of HBIG therapy.