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RAPID COMMUNICATION

Differential reactivity of mouse monoclonal anti-HBs antibodies with recombinant mutant HBs antigens

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Abstract

AIM: To investigate the reactivity of a panel of 8 mouse anti-hepatitis B surface antigen (HBsAg) monoclonal antibodies (mAbs) using a collection of 9 recombinant HBsAg mutants with a variety of amino acid substitutions mostly located within the "a" region.

METHODS: The entire HBs genes previously cloned into a mammalian expression vector were transiently transfected into COS7 cells. Two standard unmutated sequences of the ayw and adw subtypes served as controls. Secreted mutant proteins were collected and measured by three commercial diagnostic immunoassays to assess transfection efficiency. Reactivity of anti-HBs mAbs with mutated HBsAgs was determined by sandwich enzyme-linked immunosorbent assay (ELISA).

RESULTS: Reactivity of anti-HBs mAbs with mutated HBsAgs revealed different patterns. While three mutants reacted strongly with all mAbs, two mutants reacted weakly with only two mAbs and the remaining proteins displayed variable degrees of reactivity towards different mAbs. Accordingly, four groups of mAbs with different but overlapping reactivity patterns could be envisaged. One group consisting of two mAbs (37C5-S7 and 35C6-S11) was found to recognize stable linear epitopes conserved in all mutants. Mutations outside the "a" determinant at positions 120 (P→S), 123(T→N) and 161 (M→T) were found to affect reactivity of these mAbs.

CONCLUSION: Our findings could have important implications for biophysical studies, vaccination strategies and immunotherapy of hepatitis B virus (HBV) mutants.

Key words: Hepatitis B surface antigen; Hepatitis B virus; Mutant; Epitope mapping; Vaccination; Monoclonal antibody

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INTRODUCTION

In spite of the progress made in vaccine development, hepatitis B virus (HBV) infection remains a major health care problem worldwide. Upon exposure to HBV, 5%-10% of healthy adults fail to mount a protective antibody response and become chronically infected[1]. A similar proportion of healthy individuals do not respond to HB vaccination[2,3]. Lack of response has largely been attributed to parameters associated to the host immune system, including defective antigen presentation[4,5], diminished Th1/Th2 response[6,7], restricted HBsAg-specific T- and/or B-cell repertoires[8,9] and HLA-linked B-cell deletion or suppression[10,11]. Viral factors, however, particularly evolving mutant variants may also play an important role. The unique replication cycle of HBV[12] provides much opportunity for mutations to appear[13]. In addition, immune response is considered as a synergistic pressure for emergence of these mutants[13,14]. Amino acid substitutions resulting from such mutations may affect the structure of hepatitis B surface antigens (HBsAgs) that consist of three overlapping molecules, namely large (L), middle (M) and small (S) proteins[13].

The small protein, traditionally known as HBsAg, contains the immunodominant "a" determinant shared by all serotypes and genotypes of HBV, together with

serotypic epitopes determining the four major subtypes of HBV, that is *dw, ym, dr* and *yr*[15]. The "a" determinant (amino acids 124-147) is located within a large antigenic area referred to as the major hydrophilic region (MHR). Tertiary structure of this area is crucial for its recognition by the immune system. Hence, mutations within this area may allow emergence of escape mutants with reduced binding affinity to HBsAg-specific antibodies. Such mutants may also skip identification by antibody-based diagnostic assays[16,17].

Monoclonal antibodies (mAbs) against HBsAg are widely used in diagnostic immunoassays[18,19]. However, these assays may not detect all cases of infection with variant viruses carrying mutations in some critical positions of the MHR[16,18,20,21]. Emergence of mutant HBV variants due to the escape from immunological pressure, either following immunoprophylaxis with polyclonal or monoclonal anti-HBs antibody and/or vaccination with HBsAg, may hinder detection of HBsAg by the current monoclonal antibody-based immunoassays. Such mutations, predominantly accumulated within the "a" determinant, could also result in breakthrough infection in a number of vaccinated subjects[22,23]. These mutants will have selective advantage for propagation and vertical or horizontal transmission to vaccinated individuals[16,24].

Recent reports of accelerated accumulation of "a" determinant mutants in vaccinated children following an universal vaccination program[25,26], together with a high prevalence of "a" determinant mutations in natural HBsAg mutants collected from unvaccinated carriers[27], have brought serious concerns to the current vaccination policy. This has prompted the Viral Hepatitis Prevention Board of WHO to consider development of strategies to prepare for eventual increase of mutant viruses and to recommend establishment of an independent global network for appropriate monitoring of such mutants[28,29].

In the present study, we have characterized eight murine HBsAg- specific mAbs as potential tools for detection of HBsAg mutants. The results presented in this paper and similar papers reported previously[20,30,31] will help to identify the most influential mutations relevant to vaccine escape and immunodetection failure.

MATERIALS AND METHODS

Monoclonal antibodies

Eight murine mAbs were raised against a recombinant wild-type hepatitis B surface antigen (rHBsAg) of the adw subtype (Heberbiovac, Heberbiotec, Cuba) as described elsewhere[19]. Characteristics of these mAbs are summarized in Table 1.

Production, purification and biotinylation of polyclonal anti-HBs antibody

White New Zealand rabbits were administered four im injections with 10 μg of standard rHBsAg (adw) at two-week intervals. The first dose was given in complete Freund's adjuvant (Sigma, USA), whereas other doses were administered in incomplete Freund's adjuvant (Sigma, USA). Following serum titration of anti-HBs antibody from immunized rabbits, hyperimmune sera were collected and purified using a column of rHBsAg coupled to CNBr-activated sepharose-4B (Amersham Biosciences, Sweden). Purified antibody was biotinylated using biotinyl N-hydroxysuccinimide (BNHS) ester (Sigma) as previously described by Bayer *et al*[32], with some modifications. Briefly, IgG (1 mg/mL) was dialyzed overnight in sodium bicarbonate (pH 8.5). BNHS ester freshly dissolved in dimethyl sulfoxide (Merck, Germany) at 1 mg/mL was added to predialyzed IgG at a 1:10 (v/v) ratio and incubated at 25℃ for 4 h. The mixture was then dialyzed overnight against 0.1 mol/L Tris-HCl (pH 7.4) dispensed into small aliquots and stored at -20℃ to avoid frequent freezing and thawing.

Table 1 Characteristics of monoclonal anti-HBs antibodies

Clone	Isotype	Kaff (M^{-1})	Recognized epitope
34D7-S3	IgG1	5.24×10^8	Conformational
33D7-S4	IgG1	3.27×10^8	Conformational
38F3-S6	IgG1	3.75×10^8	Conformational
37C5-S7	IgG1	4×10^8	Linear
35C6-S11	IgG1	2.87×10^8	Linear
35G9-S15A	IgG1	1.73×10^8	Conformational
47G3-S15C	IgM	NI	Conformational
31D4-H12	IgG1	2.2×10^8	Linear

NI: Not identified.

Table 2 Mutations and subtype of recombinant mutant HBs antigens expressed in COS7 cells

Clone	Mutations	Subtype
Gly D	Standard sequence	adw
Gly y	Standard sequence	ayw
91-4696	S113T/T143S	adw
SA6	Q129R/G130N/A166V	adw
SA7	M133T	ayw
1056	P120S/S143L	ayw
PA17D	D144E/multiple mutations	NI
M5	Y100S/T118V/R122K/M133I/ Y134N/P142S/S143L/G145K	ayw
BA2.4	Y100C/P120T	ayw
SA4	M133T/y161F	adw
BA3.2	T123N/C124R	ayw

NI: Not identified.

Preparation of expression plasmid clones encoding HBsAg mutants

Plasmid DNA (pRK5) of nine HBsAg variants and two unmutated standard adw and ayw isolates (Table 2) were constructed as described previously[20]. Plasmid DNA was propagated by transformation of *E coli* strain DH5α (CinnaGen, Iran) by electroporation[33]. In brief, appropriate amount (depending on the clone) of plasmid DNA was mixed with electrocompetent cells, incubated on ice and subsequently pipetted into a cold electroporation

cuvette (Bio-Rad, USA). Pulse of electricity was delivered and super optimal catobolite (SOC) medium was added immediately to electroporated cells. Following 2 h incubation at 37℃, different volumes of electroporated cells were plated onto LB agar (Sigma) containing 75 μg/mL ampicillin (Sigma). Electrocompetent cells without DNA were used as a negative control. Transformed colonies were selected, tested and cultured in LB broth containing 75 μg/mL ampicillin. Plasmid DNA was subsequently purified using GIAGEN plasmid extraction kit (QIAGEN, USA).

Transfection of expression plasmids

COS7 cells (NCBI C143) provided by National Cell Bank of Iran (Pasteur Institute of Iran, Tehran) were cultured in RPMI-1640 (Gibco, USA) containing 100 mL/L heat-inactivated fetal bovine serum (Biochrom, Germany), penicillin (100 IU/mL) and streptomycin (100 μg/mL). Cells were transiently transfected using Lipofectamin 2000 (Invitrogen, USA). The amount of viral DNA was adjusted in all experiments to 0.8 μg/well in a 24-well plate (Nunc, Denmark). A subconfluent monolayer of COS7 cells was washed with growth medium without antibiotics and 500 μL of Opti-MEM I Reduced Serum Medium (Invitrogen) was added. Plasmid DNA was diluted in 50 μL of Opti-MEM I. Then 2 μL Lipofectamin 2000 pre-diluted in 50 μL of Opti-MEM I was added to diluted DNA and kept at room temperature for 20 min. The complex was added to the cells and the cells were incubated at 37℃ in a humidified atmosphere containing 50 mL/L CO_2. Following a 2 h incubation, 500 μL of complete medium was added and culture supernatant was harvested 48-96 h later. pRK5 plasmid with or without the insert carrying a standard sequence of HBV DNA [adw (Gly D) and ayw (Gly Y) subtypes] was used as negative and positive controls, respectively.

Commercial HBsAg detection kits

Supernatants of transfected cells were tested for the presence of HBsAg using three different sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's guidelines. The Bioelisa HBsAg colour kit (Biokit, Spain) employs polyclonal Ab as the capture (coating) layer, whereas the Hepanostika HBsAg Uni-Form II (BioMerieux, The Netherlands) and the ETI-MAK-4 (Diasorin, Italy) kits employ mAb as the coating Ab. All three kits contain peroxidase-conjugated polyclonal anti-HBs antibody as detector.

Determination of reactivity of anti-HBs mAbs with HBsAg mutants by sandwich ELISA

A panel of eight mAbs was used in this study. Monoclonal anti-HBs Abs were dissolved in phosphate-buffered saline (0.15 mol/L PBS, pH 7.2) at a final concentration of 10 μg/mL. The wells of 96-well flat-bottom microtiter plates (Maxisorp, Nunc, Denmark) were coated with anti-HBs Ab (100 μL/well) and incubated for 90 min at 37℃. The wells were then washed three times with PBS and plates were blocked with PBS containing 30 g/L skim milk (Merck, Germany) for 90 min at 37℃. After washing with PBS containing 0.5 mL/L Tween 20 (PBS/T, Sigma), 100 μL of supernatant of the transfected cells was added to the plates. Following incubation at 37℃ for 90 min, the plates were washed and filled with appropriate dilution of biotinylated-rabbit anti-HBs Ab. After incubation for 90 min and washing, appropriate dilution of peroxidase-conjugated streptavidin (Sigma) was added and the reaction was then revealed with tetramethylbenzidine (TMB, Sigma) substrate. Finally, the reaction was stopped with 200 mL/L H_2SO_4 and the absorbance (A_{450}) was measured by a multiscan ELISA reader (Organon Teknika, Boxtel, The Netherlands) at 450 nm.

Table 3 Reactivity of mutant HBs antigens secreted by transfected cells as determined by three commercial ELISA kits

Expressed	Bioelisa		ETI-MAK-4		Hepanostika		
HBsAg	A_{450}	Index	A_{450}	Index	A_{450}	Index	Conc.
Gly D	> 3	> 5.5	2.9	5.1	> 3	> 11	> 200
Gly Y	2.97	5.5	2.97	5.2	> 3	> 11	> 200
91-4696	> 3	> 5.5	2.87	5	> 3	> 11	> 200
SA6	2.96	5.5	2.74	4.8	> 3	> 11	> 200
SA7	> 3	> 5.5	2.87	5	> 3	> 11	> 200
1056	1.5	2.8	1.8	3.2	2.32	8.6	162
PA17D	1	1.8	2.7	4.7	> 3	> 11	> 200
M5	1.59	2.9	0.74	1.3	1.39	5.1	92
BA2.4	1.66	3.1	2.08	3.6	2.33	8.6	162
SA4	1.67	3.1	1.34	2.3	2.12	7.8	148
BA3.2	0.54	1	0.65	1.1	1	3.7	62
pRK5	0.17	0.31	0.08	0.15	0.1	0.37	0
Medium	0.18	0.33	0.11	0.19	0.1	0.37	0

Results are expressed as absorbance (A) measured at 450 nm, index (ratio of absorbance of mutant sample to absorbance obtained for 1.25 ng/mL of standard adw HBsAg) and concentration (Conc.) presented as ng/mL. Concentration was measured only by the Hepanostika kit using the standard adw HBsAg to construct the standard curve.

RESULTS

Detection of expressed HBsAg mutants by commercial ELISA kits

Transfection efficiency of all expressed HBsAg clones, including nine mutants and two unmutated standard sequences, was measured using 3 commercial diagnostic kits. To obtain a comparable index in each assay, absorbance (A_{450}) of each sample was represented as the ratio to absorbance obtained for 1.25 ng/mL of a purified recombinant unmutated HBsAg of the adw subtype (immunogen) (Table 3). All expressed HBsAg proteins were detectable by all three assays, albeit at different levels. While the index value for 91- 4696 was similar to the standard sequences (Gly D and Gly Y), BA3.2 mutant showed the lowest index using all three assays, significantly lower than the standard sequences. Overall, the Hepanostika diagnostic kit gave higher absorbance and relative indexes implying better sensitivity compared to the other two kits. This kit was subsequently used to quantify the concentration of secreted HBsAg in culture supernatants of transfected cells by extrapolation from known input of the standard adw HBsAg (Table 3).

Table 4 Reactivity patterns of monoclonal anti-HBs antibodies with expressed HBs antigen mutants

Monoclonal antibody	GlyD	GlyY	91-4696	SA6	SA7	1056	PA17D	M5	BA2.4	SA4	BA3.2
34D7-S3	++	++	++	++	++	+	-	-	+	+	-
33D7-S4	++	++	++	++	++	+	-	-	$+^{w}$	+	-
38F3-S6	+++	+++	+++	+++	+++	+++	$+^{w}$	-	-	+	-
37C5-S7	+++	+++	+++	+++	+++	$+^{w}$	++	+++	++	$+^{w}$	+
35C6-S11	+++	+++	+++	+++	+++	$+^{w}$	+++	+	+	$+^{w}$	$+^{w}$
35G9-S15A	+++	+++	+++	+++	+++	-	-	-	$+^{w}$	$+^{w}$	-
31D4-H12	++	++	++	++	++	-	-	-	$+^{w}$	$+^{w}$	-
47G3-S15C	+++	+++	+++	+	+	-	-	+	+	-	-

Results are expressed as ratio of absorbance (*A*) of a given mutant to that obtained for 1.25 ng/mL of standard recombinant adw HBsAg and presented as : -: ≤ negative control (pRK5 plasmid); $+^{w}$: > negative control < 1; +: 1-3; ++: 3-6; +++: > 6.

Table 5 Cross inhibition of monoclonal antibodies binding to HBs antigen

Unlabeled mAbs	HRP-conjugated mAbs						
	34D7-S3	33D7-S4	38F3-S6	37C5-S7	35C6-S11	35G9-S15A	31D4-H12
34D7-S3	4	4	4	2	3	4	1
33D7-S4	3	4	4	2	4	3	2
38F3-S6	3	3	4	1	3	4	2
37C5-S7	1	1	1	4	1	2	3
35C6-S11	2	4	4	2	4	3	2
35G9-S15A	4	3	3	1	3	3	2
47G3-S15C	3	3	3	1	3	3	2
31D4-H12	2	1	1	3	1	2	3

1: 0%-20% inhibition; 2: 20%-50% inhibition; 3: 50%-80% inhibition; 4: 80%-100% inhibition.

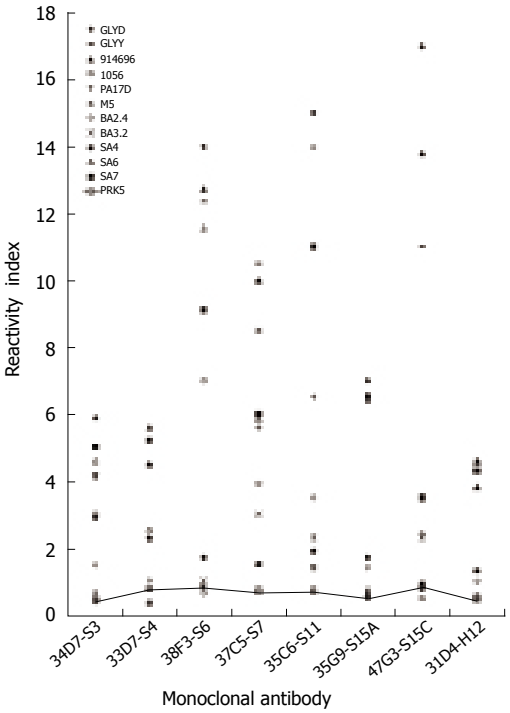

Figure 1 Reactivity of mouse monoclonal anti-HBs antibodies with recombinant HBs antigen mutants. Reactivity is expressed as relative index defined as the ratio of absorbance (*A*) of a given mutant to the absorbance obtained for 1.25 ng/mL of the standard adw HBs antigen. The horizontal line represents the results obtained for pRK5 plasmid without insert used as the negative control.

Reactivity patterns of anti-HBs mAbs to the expressed HBsAg mutants

Immunoscreening was carried out on supernatants from cells transfected with all constructs using the mAbs as the coating layer. Supernatants of untransfected cells or cells transfected with pRK5 plasmid vector alone were used as background (blank) and negative control, respectively. The results are presented in Figure 1 and summarized in Table 4. While the standard sequences (Gly Y and Gly D) and 3 mutant proteins (91-4696, SA6 and SA7) were detectable by all eight mAbs, only two of the mAbs (37C5-S7 and 35C6-S11) reacted with BA3.2 having two mutations at positions 123 and 124. The remaining mutants displayed different reactivity patterns. Cross-inhibition studies were performed to find out whether binding of an antibody could inhibit binding of other mAbs to their epitopes, enabling identification of overlapping epitopes recognized by different mAbs. The results illustrate different inhibitory patterns, partly compatible with those obtained from the direct binding assays (Table 5).

DISCUSSION

In the present study, eight mAbs raised to a recombinant unmutated HBsAg of the adw subtype were tested for their bindings to a panel of nine mutants HBsAg. We have recently demonstrated that these mAbs recognize different conformational or linear epitopes common to adw, ayw and adr subtypes, supposedly the "a" determinant[19].

This determinant is conserved in all subtypes of HBV and confers a protective antibody response to HBV infection by all subtypes[34,35]. Vaccination of healthy individuals with recombinant HBsAg has also been shown to induce an antibody response restricted to the "a" determinant[36]. Although the structure of the "a" determinant has not been thoroughly characterized and seems to encompass a large number of amino acid residues common to all subtypes of HBV, an important region of this determinant lies between residues 124 and 147. This region is a part of a highly hydrophilic area located between residues 100 and 160. Presence of 5 cystein residues at positions 124, 137, 138, 139 and 147 creates at least 2 loops protruding from the surface of

HBsAg as highly immunogenic epitopes. The majority of mAbs produced against HBsAg have been shown to recognize conformational epitopes within one of these loops[16,17,30,31]. The tertiary structure created by these disulphide bonds is critical for antigenicity of HBsAg and replacement of cysteine residues at positions 124 and 147 with serine destroys its antigenicity[37], demonstrating the conformational nature of the epitopes. Five of our mAbs reacted with conformational epitopes (Table 1), which were destroyed upon treatment of the molecule with a reducing agent, such as 2-ME.

Both type and position of substituted amino acids within the "a" determinant seem to be equally important. This is clearly reflected in the reactivity patterns of our mAbs. Thus three of the mutants including SA7, SA6 and 91-4696 were strongly reactive with all our mAbs. The reactivity indexes obtained for some of these mutants, particularly 91-4696, were similar to/or higher than those obtained for the two standard sequences (Figure 1). They also displayed strong reactivity with the three commercial diagnostic kits employed in this study. Similar reactivity patterns have already been reported for these three mutants, using a variety of commercial diagnostic kits and HBsAg-specific mAbs[20,30]. Altogether six replacement mutations, including S113T/T143S (91-4696), Q129R/G130N/A166V (SA6) and M133T (SA7), were identified in these three mutants, none of which seemed to be crucial for HBs antigenicity. Of the two substitutions occurring at positions 113 and 143 in the 91-4696 mutant, the latter is more relevant to antigenicity of the "a" determinant. However, replacement of threonine with serine at this position has also been observed in sequences from a number of unmutated isolates of HBV deposited in the GenBank. This may explain the preserved binding activity of this mutant with all our mAbs. Surprisingly, replacement of serine with leucine at the same position in another mutant (1056) resulted in either substantial reduction or complete loss of reactivity towards most of the mAbs. Three of the mAbs (35G9-S15A, 31D4-H12 and 47G3-S15C) were completely negative and two mAbs (37C5-S7, 35C6-S11) were weakly positive (Figure 1 and Table 4). This mutant, however, contains another mutation at position 120 leading to replacement of proline with serine. This mutation could have a significant effect on reactivity of some mAbs[30,38] and has also been reported as a vaccine escape mutant with reduced binding affinity to anti-HBs Ab[13]. To distinguish whether the P120S or S143L mutation is responsible for diminished reactivity of the 1056 mutant, we looked at binding patterns of M5 mutant. This mutant contains an S143L mutation together with 7 more mutations, mostly located within the first and second loops of "a" determinant, but not the P120S substitution. Reactivity of 3 of the 1056 non-reactive or low-reactive mAbs (37C5-S7, 35C6-S11 and 47G3-S15C) was restored, suggesting direct or indirect influence of this residue on their binding activity. Since the M5 mutant contains 8 substitutions, a definite conclusion cannot be drawn from this comparison. Ideally, a mutant with a single mutation at P120 is required to study the influence of this residue.

Altogether, from the reactivity patterns observed in this study, our mAbs could be broadly classified into four distinct, but overlapping groups (Table 4). The first group includes 34D7-S3, 33D7-S4 and 38F3-S6 mAbs. Reactivity of these mAbs with the 1056 mutant distinguished this group of mAbs from other groups. None of these mAbs reacted with PA17D, M5 and BA3.2 mutants, carrying multiple mutations extending from residue 100 to 145. Lack of reactivity with BA3.2 (T123N/C124R) that has a cysteine to argenine substitution at position 124 could largely be attributed to low immunoreactivity or a low secretion rate of this mutant from transfected cells (Tables 3, 4). This mutation resulted in disruption of the disulphide bond of the first loop of "a" determinant with drastic conformational changes leading to retention of the molecule in transfected cells. Complete lack of secretion of this mutant by transfected COS7 cells has already been reported by two groups[20,30] who demonstrated the presence of low levels of BA3.2 molecule in cell lysate, but not in the culture supernatant of transfected cells. In the present study, however, BA3.2 was readily detected in culture supernatant of the transfected cells by three diagnostic kits, as well as two of mAbs (37C5-S7 and 35C6-S11). Loss of reactivity of the first group of mAbs (34D7-S3, 33D7-S4 and 38F3-S6) with BA3.2 seems to be primarily affected by conformational changes due to disruption of the disulphide bond. Indeed, these mAbs recognize highly conformational overlapping epitopes, as judged from the Western blot (data not shown) and cross-inhibition results (Table 5).

The BA3.2 reactive mAbs (37C5-S7 and 35C6-S11) constitute the second group of mAbs. Reactivity of 37C5-S7 and, to a lesser extent, 35C6-S11 with BA3.2 could be due to the linear nature of the epitopes recognized by both mAbs. However, the reduced binding activity of these antibodies with BA3.2 seems to be affected by the T123N mutation. Furthermore, recognition of three other mutants, namely PA17D, M5 and BA2.4 having multiple mutations, by these two mAbs makes a distinction between this group of mAbs with other groups. A variety of mutations exist within the first and second loops of "a" determinant of these mutants, including residues 133, 134, 144 and particularly 145, which are critical for binding many of the mAbs[31]. These mutations, however, do not significantly influence reactivity of 37C5-S7 and 35C6-S11 mAbs. Instead, a few mutations located outside these loops at positions 120 (1056), 123 (BA 3.2), 133 and 161 (SA 4) induced a significant reduction of reactivity of 37C5-S7 and 35C6-S11 mAbs with the corresponding mutants. The reduced reactivity observed for both mAbs with 1056 (P120S, S143 L) and SA 4 (M133 T/Y 161F) mutants does not seem to be related to replacements of 143 and 133 residues, because the same mutations are found in 91-4696 and SA7 mutants which strongly bind to both mAbs (Table 4). Therefore, the P120S, T123 N and M161T mutants are suspected to contribute to formation of the linear epitopes recognized by 37C5-S7 and 35C6-S11 mAbs. Amino acid 161 has been proposed to be located at the surface of HBsAg[39] which could potentially influence antigenicity of the molecule. Such escape and natural mutants with altered antigenicity have already been reported[40,41]. Deletion mutagenesis and phage display technology may help to

map these epitopes more precisely.

The third group of mAbs includes only one member, that is 47G3-S15C which cannot bind to either of four mutant proteins (1056, PA17D, BA3.2 and SA4), but reacts with M5 and BA2.4 mutants. The most influential mutations are thought to be P120S, T123N and Y161F similar to the pattern predicted for the second group of mAbs. However, unlike 37C5-S7 and 35C6-S11 mAbs, 47G3-S15C does not bind to the PA17D mutant. This molecule has been reported to carry a mutation at position 144 (D→E), as well as many other undisclosed residues[30], making it difficult to assign the contributing residues. Involvement of D144E mutation seems to be unlikely, since this mAb reacts with the M5 mutant having multiple mutations at both sides of this amino acid, including P142S, S143L and G145R. This mAb may recognize a conformational epitope within the "a" determinant which is affected by mutations outside this region. Interestingly, mutations at residues close to the "a" determinant, particularly at positions 120 (P→Q or P→S) and 123 (T →N), were found to have a significant impact on the conformation and antigenicity of the "a" region epitopes leading to loss of reactivity of some "a" determinant-specific mAbs[30,38].

The fourth group of mAbs is comprised of 35G9-S15A and 31D4-H12 antibodies. Both mAbs did not react with four mutants (1056, PA17D, M5 and BA3.2) and reacted weakly with two other mutants (BA2.4 and SA4). Although the epitopes recognized by 35G9-S15A and 31D4-H12 are structurally different (conformational and linear, respectively), they overlap to some extent (Table 5), suggesting their close spatial location on HBsAg. Considering the number of mutated residues in non-reactive and low-reactive mutants, assignment of the contributing amino acids and localization of the corresponding epitopes could not be predicted with certainty. Employment of mutants with overlapping mutations at one or two amino acids covering the entire major hydrophilic region and particularly the "a" determinant will help to map the epitopes and to determine the crucial amino acids involved in interaction with our anti-HBs mAbs.

In summary, our results could have important clinical implications for immunoscreening and diagnosis of HBV infection and design of new generation of recombinant HB vaccines. Although recent epidemiological survey failed to demonstrate close association between breakthrough infection and mutant HBV variants in vaccinated children in Taiwan[26], incorporation of such mutants within the current standard recombinant HBs vaccines may later be considered as the new generation vaccines to prevent transmission of HBV mutants.

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