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Mutant hepatitis B virus surface antigens (HBsAg) are immunogenic but may have a changed specificity

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Abstract

Mutant hepatitis B virus with substitutions within the coding region for HBV surface antigen (HBsAg) has been found naturally in chronic carriers. It is therefore important to clarify whether the identified substitutions within the HBsAg have impact on the antigenicity and immunogenicity of HBsAg. A total of nine mutated HBV s-genes with single representative mutations were generated by site-directed mutagenesis and subcloned into an expression vector. The binding of polyclonal and monoclonal antibodies to these mutant HBsAg (mtHBsAg) was tested by immunofluorescence (IF) staining of cells transfected with the expression vectors. The amino acid (aa) substitutions like G145R, F134S, and C147W affected the binding of anti-HBs antibodies to corresponding mtHBsAg to different extents. The impact of aa substitutions G145R and F134S on the immunogenicity was accessed by genetic immunization of mice with vectors expressing middle HBsAg with the corresponding mutations. The immunized mice developed antibodies to recombinant HBsAg containing the HBV preS region and HBsAg-specific cytotoxic T-cell. However, the development of antibody response to wild-type small HBsAg was significantly impaired by the aa substitutions in HBsAg. Based on this fact, we further investigated whether the mtHBsAg with the aa substitution G145R is able to induce mutant-specific antibody responses. Strikingly, serum samples from mice immunized with mtHBsAg with G145R recognized plasma-derived mtHBsAg. Two mouse MAbs specific to mtHBsAg were generated. One MAb recognized mtHBsAg with G145R but not wild type and other mtHBsAg. We conclude that HBsAg with aa substitutions are immunogenic but may have a changed fine specificity.

Keywords: Hepatitis B; Immunogenic; Surface antigen

Introduction

Hepatitis B virus (HBV) is an enveloped virus and causes acute self-limited and chronic infections in human (Hollinger and Liang, 2001). Chronic HBV infection is one of the major causes for liver diseases, cirrhosis, and hepatocellular carcinomas. HBV infection can be prevented by vaccination with recombinant hepatitis B surface antigens (HBsAg) (Szmuness et al., 1981). HBsAg induces specific anti-HBs antibodies that are mainly directed to the central region of HBsAg termed as a-determinant (Bhatanagar et al., 1985; Brown et al., 1984; Chen et al., 1996; Schirmbeck et al., 2001; Zheng et al., 2002). However, HBV mutants with changed surface antigens are able to escape immune control

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(for reviews, see Carman, 1997; Cooreman et al., 1999; Gunther et al., 1999). HBV escape mutants were found first in children immunized with HBsAg (Carman et al., 1996; Karthigesu et al., 1994). Though they developed anti-HBs antibodies at the protective level, persistent HBV infection occurred in some individuals. HBV mutants may emerge and cause infection in liver transplant patients and infants born to HBV carrier mothers receiving hyperimmunoglobulin against HBV as immunoprophylaxis (Carman, 1997; Carman et al., 1999; Chiou et al., 1997; Ghany et al., 1998; He et al., 1998; Hsu et al., 1997; Lee et al., 1997; Ni et al., 1995; Protzer et al., 1998; Sterneck et al., 1997; Waters et al., 1992). The sequence analysis of HBV isolates from these patients revealed that the HBsAg carries amino acid (aa) substitutions within the a-determinant. These aa substitutions reduce the binding affinity of HBsAg to specific antibodies and probably enable HBV to escape the neutralization by anti-HBs antibodies (Chiou et al., 1997; Cooreman et al., 2001; Grethe et al., 1998; Ireland et al., 2000; Lu and Lorentz, 2003; Seddigh-Tonekaboni et al., 2000; Waters et al., 1992).

Sequences of natural HBV isolates show a great variability (Gunther et al., 1999; Norder et al., 1993). HBV isolates carrying substitutions within the HBsAg a-determinant were found to occur in chronic HBV-infected patients, such with anti-HBc antibody as the only marker (Carman et al., 1997; Grethe et al., 1998; Hou et al., 2001; Kohno et al., 1996; Oguru et al., 1999; Weinberger et al., 2000; Yamamoto et al., 1994). The presence of HBV mutants in the general population imply that they circulate naturally; thus, they might cause acute and chronic HBV infection, particularly in some individuals like immune compromised patients despite of the immunoprophylaxis. However, the influence of these identified aa substitutions of HBsAg on the antigenicity and immunogenicity of HBsAg is not obvious. The recent approach to define the antigenicity of mutant HBsAg (mtHBsAg) is to use monoclonal anti-HBs antibodies to detect mutant HBsAg in immunofluorescence (IF) staining, as demonstrated in several studies (Chiou et al., 1997; Cooreman et al., 2001; Torresi et al., 2002). It has been shown that the binding of monoclonal antibodies to HBsAg may be affected by aa substitutions within the a-determinant. Further, we and other groups explored the genetic immunization as a tool to study the immunogenicity of wild type HBsAg (wtHBsAg) and mtHBsAg (Lu and Lorentz, 2003; Schirmbeck et al., 2003; Wu et al., 1999; Zheng et al., 2002). The results of these studies indicate that the a-determinant is the only immunogenic region on the HBsAg polypeptides. The mtHBsAg have a reduced ability to induce anti-HBs antibodies while the induction of HBsAg-specific cytotoxic T lymphocytes was not impaired (Lu and Lorentz, 2003). Thus, suitable methods are available to judge the antigenicity and immunogenicity of mtHBsAg.

Two important issues about mtHBsAg are not studied in detail up to date. First, aa substitutions within the HBsAg occur usually at many positions (Weinberger et al., 2000). The significance of single aa substitutions on the antigenicty and immunogenicity is often not clear. Second, mtHBsAg are immunogenic since they are able to induce normal CTL responses (Lu and Lorentz, 2003). Though they often failed to induce a detectable anti-HBs antibody response, they may possibly prime antibody response with a changed specificity. In the present study, we focused on these two topics. A total of nine single mutations found in patients with anti-HBc antibody as the only marker for HBV infection was selected and introduced into a HBsAg subtype adw2 sequence by site-directed mutagenesis. The influence of these specific aa substitutions on the antigenicity of HBsAg was examined by transient expression and IF staining with polyclonal and monoclonal antibodies (MAb) to HBsAg and with purified immunoglobulins (IgGs) from human anti-HBs positive sera. Particular attention was paid to mtHBsAg with aa substitutions G145R and Y134S. The ability of these two different mtHBsAgs to induce specific antibody and CTL responses was assessed by genetic immunization in mice. To differentiate the specificity of antibodies induced by genetic immunization, ELISAs using different antigens HBsAg, middle HBsAg (MHBsAg) with the preS domain, and mtHBsAg with aa substitution G145R were performed with sera of immunized mice. The induction of CTLs to the H-2Ld restricted epitope on HBsAg aa 29–38 in BALB/cJ (H-2d) mice were assessed by in vitro expansion and staining of specific CTLs. Finally, MAbs specific to mtHBsAg with G145R were generated by a combination of plasmid and protein immunization for further analysis of the fine specificity of the antibody response to mtHBsAg.

Results

Reduced binding of antibodies to HBsAg a-determinant to HBsAg with single mutations

Previously, a series of mutations within the a-determinant of HBsAg were identified by sequencing of HBV isolates from patients with anti-HBc as only marker for HBV infection (Weinberger et al., 2000). Some of these mutations had been described early (Gunther et al., 1999). In addition, novel mutations within or adjacent to the a-determinant like T125M, K141Q, and P153A were identified. These mutations represent nonconservative aa substitutions and therefore may have influence on the conformation of HBsAg a-determinant and the binding of HBsAg-specific antibodies. To investigate the influence of these mutations on the antigenicity and immunogenicity of HBsAg, PCR-based mutagenesis was performed to introduce nine selected single mutations into the a-determinant of an adw2 WT sequence (pHBV991-12-1, GenBank accession number X51970). The mutated sequences were recloned into pcDNA3 and placed under the control of a cytomegalovirus immediate–early promoter (Fig. 1).

To examine whether the mutations within the HBsAg lead to the alteration of binding of HBsAg to specific anti-

HBsAg antibodies, various cell lines including hepG2 cells were transiently transfected with vectors expressing mtHBsAg and wtHBsAg and stained with a polyclonal anti-HBs and different monoclonal anti-HBs antibodies. In addition, IgGs purified from human anti-HBs positive sera were tested for their reactivity to mtHBsAg by IF staining. The representative results of IF staining with anti-HBs antibodies are summarized in Fig. 2.

WtHBsAg was clearly stained with polyclonal and a monoclonal anti-HBs antibody (Dako clone 3E7) (Fig. 2). Three of nine mtHBsAg T125M, P127T, and P153A were stained with both polyclonal and monoclonal antibodies like wtHBsAg. Obviously, many aa substitutions on the HBsAg do not affect the binding of specific anti-HBs antibodies. The staining of mtHBsAg F134S and S154P with polyclonal anti-HBs antibodies was comparable with the staining of the wtHBsAg, while staining with monoclonal antibodies resulted into a reduced intensity (Fig. 2). Thus, these mutations led to a reduced binding to antibodies with a defined specificity. The mutation at the aa position 147 of HBsAg led to a loss of a cystein residue that is involved in the intermolecular disulfide bond formation (Mangold et al., 1995). The mtHBsAg C147W was stained by polyclonal anti-HBs but failed to be detected in a commercial HBsAg ELISA (Enzygnost HBsAg 5.0, Dade Behring). The monoclonal anti-HBs antibody failed to recognize HBsAg C147W. mtHBsAg K141Q and S154V showed a reduced staining with both polyclonal antibodies and MAb. The mtHBsAg G145R were not recognized by IF staining with both polyclonal and monoclonal antibodies. The aa residue at the position 145 of HBsAg has been identified as a critical residue for antibody response (Carman et al., 1990). However, the mtHBsAg could be stained with a high-affine anti-HBs antibody (see below). Thus, some specific mutations at the critical positions within the HBsAg sequences may change the antigenicity of HBsAg.

The reactivity of antibodies to HBsAg in human subjects to wt- and mtHBsAg was tested (Fig. 2). Though many human sera had high titers of anti-HBs antibodies, IF staining of HBsAg expressing cells using these samples resulted frequently into strong unspecific staining. The purification of IgG fractions from sera reduced the unspecific staining. However, only few IgG preparations gave positive staining of transfected cells, as shown in Fig. 2. The IgG preparations from sera from two different individuals recognized normally the mtHBsAg with aa substitutions within the first half of the a-determinant and stained the mtHBsAg K141Q, C147W, and S154V with reduced intensity. The mtHBsAg 145R was not recognized. Thus, the majority of the tested aa substitutions within the second loop of the HBsAg a-determinant appeared to reduce the binding of antibodies to HBsAg.

The mutations G145R and F134S within the a-determinant of HBsAg impaired the ability to induce the anti-HBs antibody

The aa substitutions like G145R and F134S affect the antigenicity of HBsAg in different extents. However, it is unknown whether mtHBsAg with these aa substitutions retain the ability to induce anti-HBs antibody response. As demonstrated in our previous studies, genetic immunizations in the mouse model represent a useful tool to test the ability of the mtHBsAg to induce HBsAg-specific antibodies (Lu and Lorentz, 2003). Genetic immunizations of mice with plasmids expressing MHBsAg induce antibodies to the HBV

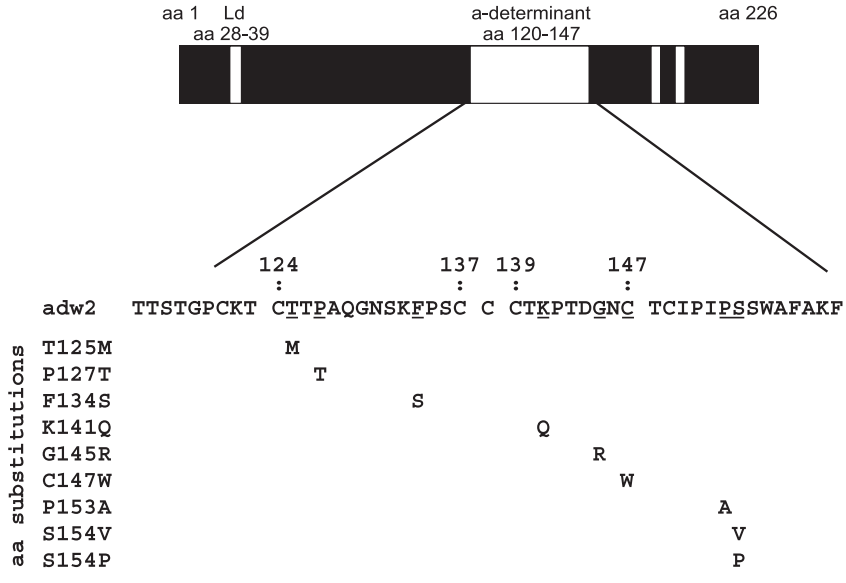
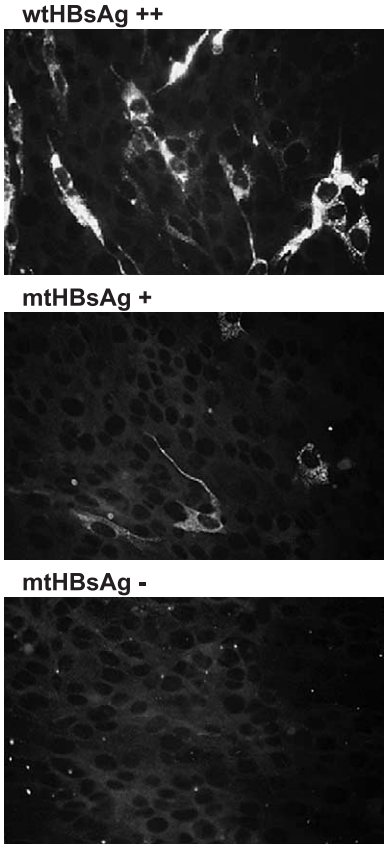


Fig. 1. Top: Map of antigenic regions within the HBsAg. The a-determinant and the murine H-2L^d-restricted CTL epitope aa 28–39 (Ld) of HBsAg are indicated. Bottom: The sequence aa 115–173 of HBsAg. The aa sequence of HBsAg from aa 115–173 of the HBV subtype adw2 was given as the reference (Norder et al., 1993). For mutant sequences, the positions and the nature of the aa substitutions introduced into the wtHBsAg sequence were indicated. The changed aa within the wild type adw2 sequence was underlined.



IF staining of transfected cells expressing wtHBsAg and mtHBsAg

HBsAg	IF staining with Polyclonal Ab	MAb clone 3E7	Human anti-HBs IgG P4
WT	++	++	++
T125M	++	++	++
P127T	++	++	++
F134S	++	+	++
K141Q	+	+	+
G145R	-	-	-
C147W	++	-	+
P153A	+	++	++
S154V	+	+	+
S154P	++	+	++

Fig. 2. IF staining of wtHBsAg and mtHBsAg with HBsAg-specific antibodies. Cells were transfected with plasmids expressing wtHBsAg or mtHBsAg, as indicated in the figures. After 48 h, cells were fixed with 50% methanol and stained with the anti-HBs antibodies. Polyclonal goat anti-HBs IgG to plasma-derived HBsAg (Biotrend) and an anti-HBs MAb (Dako, clone 3E7) were used for IF staining. Human anti-HBs IgG P4 was purified from serum of an individual received immunization with recombinant HBsAg. The intensities of the IF staining were judged by comparison with the staining of wtHBsAg. ++: strong IF staining, as seen for wtHBsAg; +: weak staining; −: no specific staining. Three representative IF staining of wtHBsAg and mtHBsAg with MAb 3E7 are shown (magnification 400×). The results of IF staining of nine mtHBsAg and wtHBsAg are listed.

preS region and the a-determinant (Schirmbeck et al., 2001; Zheng et al., 2002). Therefore, the coding sequence for the HBV preS2 region was added to both mutated s sequences to generate pS2-HBsAg145R and pS2-HBsAg134S using the previously cloned sequence (Zheng et al., 2002). This procedure resulted into plasmid expression of MHBsAg with aa substitutions G145R and Y134S within the S domain. Since the HBV preS2 region harbors potent B-cell epitopes like aa 14–32 (Neurath et al., 1984), it serves as an internal control for the suitability of the plasmids of genetic immunizations. As expected, these expression vectors encoded the MHBsAg that could be detected by polyclonal anti-HBs antibodies (data not shown).

Four groups of five mice each were immunized twice with plasmids pHBsAg-WT, pHBsAg145R, pS2-HBsAg145R, and pS2-HBsAg134S, respectively, in a time interval of 3 weeks. The immunization with pHBsAg-WT induced anti-HBs antibodies in four of five mice. Only one of five mice immunized with pHBsAg145R developed anti-HBs antibody. Immunizations with pS2-HBsAg145R and pS2-HBsAg134S did not induce any detectable anti-HBs antibody (Fig. 3A), though antibodies to MHBsAg were induced in three and four of five mice, respectively (Fig. 3B). Thus, these aa substitutions within the a-determinant affected specifically the immunogenicity of the mtHBsAg.

Though the induction of wtHBsAg-specific antibodies was impaired, genetic immunizations with plasmids expressing mtHBsAg induced normal HBsAg-specific CTL responses in mice. All immunized mice developed CTLs to HBsAg without an obvious difference between these different groups. The CTLs to the immunodominant, H-2L^d-restricted epitope on HBsAg aa 29–38 was analyzed for each expanded culture (Table 1). Forty-one percent to 75.5% of total cell counts in cultures after 7 days were positively stained with anti-CD8 MAb. 28.7% to 64.2% of CD8+ cells were stained with H-2L^d:Ig dimeric protein loaded with HBsAg aa 29–38. Dimeric proteins loaded with a control peptide aa 208–215 stained less than 4.5% of CD8+ cell counts that are supposed to be unspecific staining. Mice immunized with control plasmids like pcDNA3 did not develop any HBsAg-specific CTLs (data not shown). Similar results were obtained by using standard Cr-release assay (data not shown). These results were consistent with our previous findings with other mutant HBsAg (Lu and Lorentz, 2003).

Detection of specific antibodies direct to mtHBsAg with the aa substitution G145R

The a-determinant is the only region with the ability to induce HBsAg-specific antibodies in genetic immunizations (Schirmbeck et al., 2001; Zheng et al., 2002). Thus, aa substitutions within the a-determinant may change the specificity of the antibody responses. Particularly, the substitution G145R is assumed to affect the conformation of HBsAg a-determinant. Consequently, such mtHBsAg may induce antibodies with a changed specificity that do not efficiently bind to the wtHBsAg. To test the hypothesis, we attempted to detect the specific antibodies to mtHBsAg in sera from immunized mice. MtHBsAg was purified from serum of a patient infected with HBV mutant with an aa substitution G145R within the a-determinant and coated to the microtiter plates for ELISA. Sera from mice immunized with pHBsAg145R or pS2-HBsAg145R showed a significant reactivity to purified mtHBsAg, whether or not they were reactive to MHBsAg (Fig. 3C). The reactivity to the mtHBsAg was clearly measured at dilutions up to 1:200 of sera. In contrast, sera from pS2-HBsAg134S immunized mice or control sera did not react at a dilution of 1:25. Thus, the aa substitution G145R did not abolish the immunogenicity of mtHBsAg but appeared to change the antibody specificity.

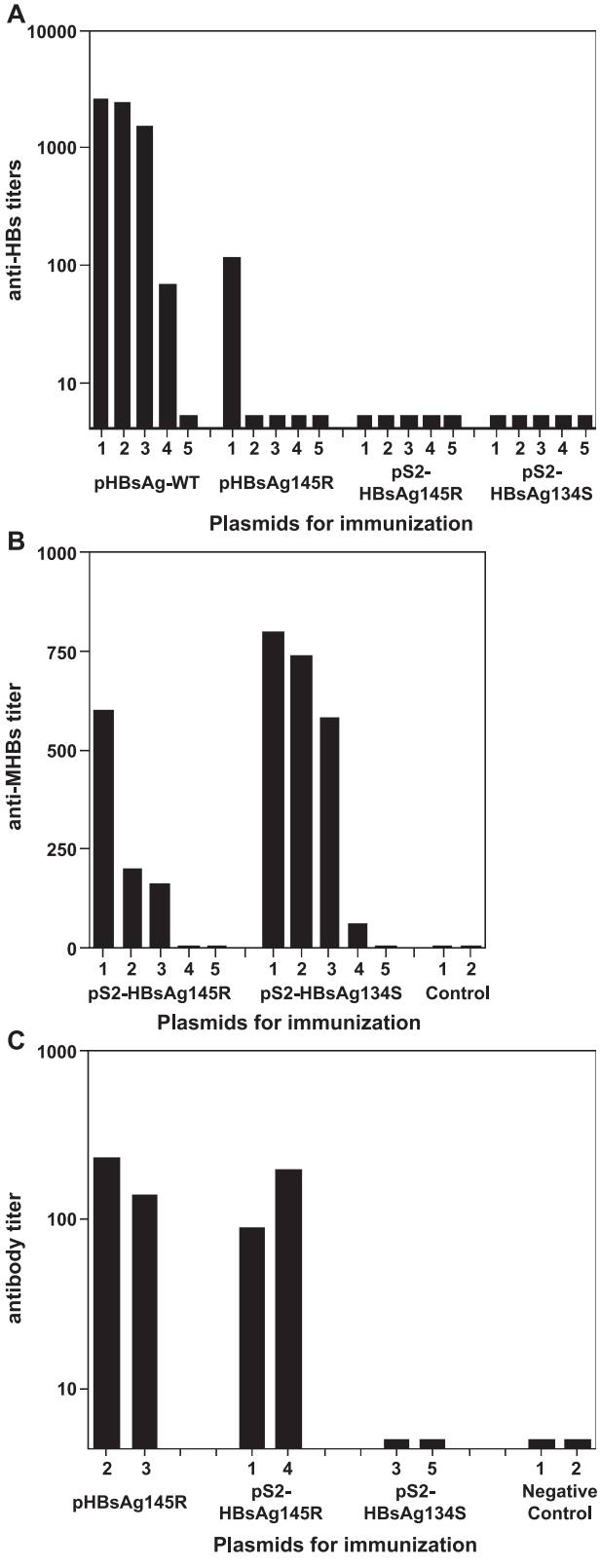


Fig. 3. Induction of anti-HBs antibodies and HBsAg-specific CTLs in mice by DNA immunization. BALB/cJ (H-2^d) mice were immunized with plasmids expressing wtHBsAg and mtHBsAg and were sacrificed three weeks after the last immunization. Sera were collected to test the presence of antibodies to wtHBsAg and mtHBsAg. (A) Sera from immunized mice were tested by a commercial kit Enzygnost anti-HBs II (Dade Behring). The anti-HBs titers were given for each individual mouse. (B) Sera from mice immunized with pS2-HBsAg145R and pS2-HBsAg134S were tested in ELISA for their reactivity to MHBsAg. Recombinant MHBsAg was used to coat the ELISA plates. The anti-HBs titers were given for each individual mouse. (C) Detection of specific antibodies to the mtHBsAg with G145R. Plasma-derived HBsAg from a patient carrying mtHBsAg G145R was used to coat the ELISA plates. Two representative samples from each group were tested due to the shortage of the mtHBsAg G145R.

Generation of specific MAb to mtHBsAg

Based on our previous results, it should be possible to generate antibodies that specifically react to mtHBsAg but bind to wtHBsAg with a reduced ability. Two groups of mice were immunized twice with pcDNA3-101 or pcDNA3-146 (Fig. 4A). The plasmid pcDNA3-146 was chosen for generation of MAbs because of the availability of the corresponding recombinant mtHBsAg for boost and screening of specific MAbs (Weber et al., 2001). Five mice of the control group received immunizations with pcDNA3-101 expressing wtHBsAg and developed anti-HBs antibodies (data not shown). Similar to the previous experiments, only one mouse developed anti-HBs antibodies after two immunizations with pcDNA3-146. This mouse received an additional boost with 800 ng of recombinant mtHBsAg-146. Hybridoma cell lines were generated with splenocytes from this mouse. Supernatants of two hybridoma cell lines 2.001.2 and 2.001.3 showed reactivity to mtHBsAg-146 (Fig. 4B). The MAbs 2.001.2 and 2.00.3 were of subtypes IgG2b and IgG3, respectively. Interestingly, MAb 2.001.2

Table 1
Induction of CD8+ cells directed to the immunodominant CTL epitope aa 28–39 of HBsAg by plasmid immunization of BALB/cJ (H-2^d) mice

Plasmids	CD8+ cells/total cell counts (%)	CD8+ cells stained with specific dimer (%)[a]	CD8+ cells stained with control dimer (%)[b]
wtHBsAg adw			
1	41.4	34.1	0.5
2	61.0	28.7	2.9
mtHBsAg G145R			
1	58.1	50.7	3.3
2	70.7	55.9	4.2
3	75.5	64.2	4.2

BALB/cJ (H-2^d) mice ware immunized with plasmids expressing wtHBsAg and mtHBsAg G145R and were sacrificed 3 weeks after the last immunization. Splenocytes from mice were prepared and expanded for 7 days in the presence of P815 cells expressing wtHBsAg. Staining of expanded splenocytes was performed with antibodies or H-2L^d:Ig dimeric protein, as indicated. The splenocytes were derived from two and three mice immunized with wtHBsAg or with mtHBsAg G145R, respectively. The staining of splenocytes was analyzed by flow cytometry.

[a] The specific H-2L^d:Ig dimeric protein was prepared by using the peptide aa 29–38 derived from HBsAg. The percentages were calculated with CD8+ cell numbers as the baseline.

[b] The control H-2L^d:Ig dimeric protein was prepared by using the peptide aa 208–215 derived from HBsAg. The percentages were calculated with CD8+ cell numbers as the baseline.

did not recognize wtHBsAg while 2.001.3 was weakly reactive to the wtHBsAg. This result provided evidence that the mtHBsAg145R was indeed able to induce mutant-specific antibody.

To test whether MAb 2.001.2 is able to recognize other mtHBsAg, 2.001.2 was purified from the hybridoma cell supernatant and used for IF staining. HepG2 cells were transfected with pS2-HBsAg145R and pS2-HBsAg134S. IF staining was performed with three MAbs, a high-affinity anti-HBsAg antibody *MAb-No2* (Lu and Lorentz, 2003), the anti-HBsAg antibody 3E7, and 2.001.2. Both mtHBsAg were recognized by the high-affine anti-HBs antibody (Figs. 5A,D). The anti-HBs antibody clone 3E7 showed no staining for S2-HBsAg145R but weak staining for S2-HBsAg134S, as tested previously (Figs. 5B,E). 2.001.2 recognized S2-HBsAg145R but did not stain S2-HBsAg134S (Figs. 5C,F). IF staining of other six different mtHBsAg with 2.001.2 was also negative (data not shown). These results clearly demonstrated that this MAb to HBsAg145R was highly specific and did not recognize other mtHBsAg.

Discussion

Though many aa substitutions within the HBsAg a-determinant are identified in HBV isolates, the majority of single aa substitutions appear to have a limited influence on the antigenicity of HBsAg. In addition, the imunogenicity of HBsAg is not necessary to be impaired if aa substitutions occur within the HBsAg a-determinant and reduce the binding of anti-HBs antibodies. Many mtHBsAg were able to induce normal anti-HBs antibodies by genetic immunization (our unpublished results). The mtHBsAg G145R is of particularly interesting since this aa substitution occurs often in patients and has a strong influence on the HBsAg antigenicity. In this paper, we clearly demonstrated that the mtHBsAg G145R was immunogenic and was able to induce mutant-specific antibody response in mice. The HBsAg a-determinant is the only immunogenic region on the small wtHBsAg polypeptide and possesses complex conformational epitopes. Therefore, aa substitutions within the a-determinant lead to conformational changes and affect the antigenicity and immunogenicity in different extent. Some aa substitutions like G145R result presumably into a significant conformational change of the HBsAg a-determinant. This conformational change does not simply lead to the loss of immunogenicity, but creates a new specificity. The mtHBsAg G145R retained partly the ability to induce antibodies to wtHBsAg. Few mice immunized with plasmids expression mtHBsAg G145R developed detectable anti-HBs antibody titers. In addition, one MAb 2.001.3 showed a cross-reactivity to wtHBsAg and mtHBsAg G145R. Consistently, infection of chimpanzees with a HBV mutant isolate carrying the mutation G145R induced anti-HBs antibody response (Ogata et al., 1997). MAb

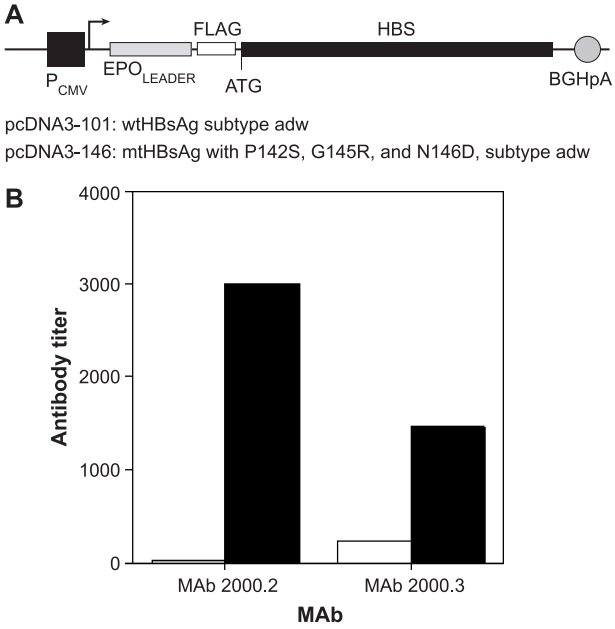


Fig. 4. Generation of specific monoclonal antibodies to mtHBsAg with the aa substitution G145R. (A) Expression vectors. Two expression vectors for wtHBsAg and mtHBsAg were generated on the basis the vector E2-TM8 (Tacke et al., 1997) and were used for genetic vaccination of BALB/cJ (H-2^d) mice. (B) Selection of monoclonal antibodies by ELISA. Recombinant wtHBsAg and mtHBsAg were used test the reactivity of monoclonal antibodies (Weber et al., 2001). The culture supernatants of hybridomas were tested for the reactivity of monoclonal antibodies. White bar: antibody to wtHBsAg; black bar: antibody to mtHBsAg HB-146.

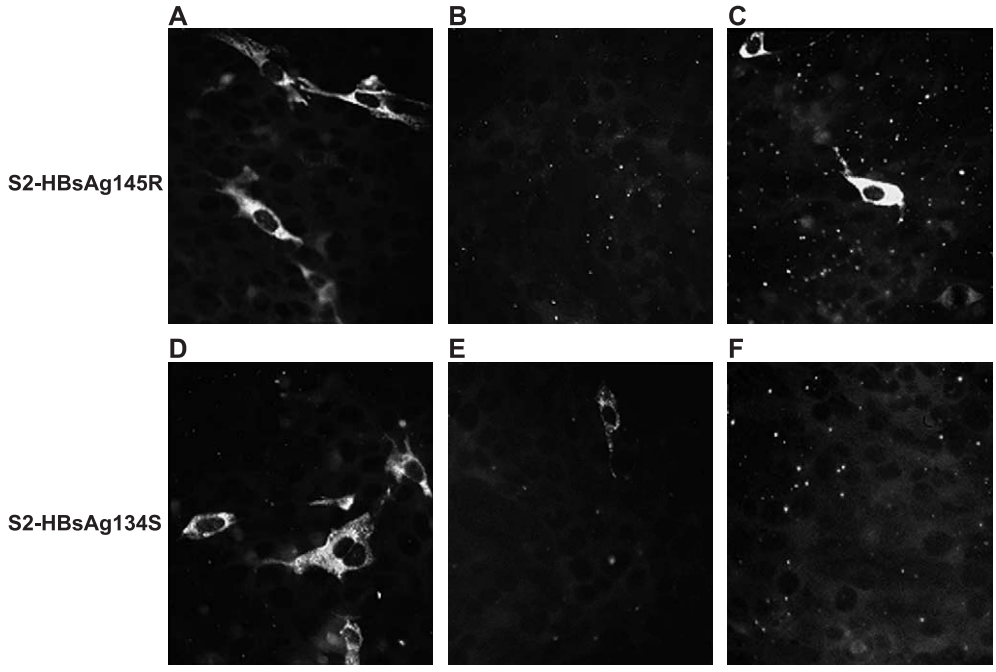


Fig. 5. IF staining of wtHBsAg and mtHBsAg with MAbs. HepG2 cells were transfected with plasmids expressing wtHBsAg or mtHBsAg, as indicated in the figures. After 48 h, cells were fixed with 50% methanol and stained with the anti-HBs MAbs. (A–C) pHBsAg145R; (D–F) pHBsAg134S. For IF staining, three different mouse MAbs were used: A and D with a high-affinity anti-HBs MAb-No2, B and E with anti-HBs clone 3E7, and C and F with MAb 2000.2. Magnification, 400×.

2.001.2 recognized the mutant HBsAg with the substitution G145R but not that with F134S and others. Thus, the MAb 2.001.2 is directed to the epitope involving the aa substitution G145R.

In our study, the majority of mtHBsAg with single aa substitutions was recognized by anti-HBs antibodies. Thus, aa substitutions do not generally abolish the binding of anti-HBs antibodies to the mtHBsAg. Only few aa substitutions strongly affected the recognition of mtHBsAg by anti-HBs antibodies. A large number of natural HBV isolates with aa substitutions within the HBsAg a-determinant were described. Multiple aa substitutions may occur often in one isolate and may contribute collectively to the reduction of antibody binding. In our previous study, we found two coexisting HBsAg mutants K29 and K30 that differ in one aa residue at the position 144. The binding of certain anti-HBs MAbs to mtHBsAg appeared to be influenced by the aa substitution at position 144, though this aa substitution itself did not obviously affect the overall antigenicity of HBsAg (Lu and Lorentz, 2003).

The infection with mutant HBV in human is still not investigated in details. HBV mutants may infect unvaccinated persons (Oon et al., 2000). Since HBV vaccines induce T- and B-cell responses, HBV may be not able to infect immunized persons only by escaping from antibody responses, as shown by the experimental infection in chimpanzees (Ogata et al., 1999). Mutant HBV may get a chance to cause acute and chronic infection in individuals with impaired cellular immune response, for example, by immune suppression or AIDS. It is not yet investigated how mutant HBV infect young vaccinees, as described in different studies (Hino et al., 2001; Karthigesu et al., 1994). Newborns may have a not fully functional T-cell response and are susceptible to infections with mutant HBV despite combined active and passive vaccinations against HBV.

In addition, the impact of the natural variation of HBV on T-cell responses in humans is not investigated. The natural HBV isolates has a great genetic variability through the whole genome (reviewed by Gunther et al., 1999). According to the sequence information, HBV isolates are divided into six major subtypes. The sequence diversity between subtypes is found to be greater than 15%. Since the currently available vaccines will only induce an immune response targeting a limited spectrum of T- and B-cell epitopes, HBV isolates of different genotypes will not be controlled with the equal efficiency. Recently, Schirmbeck et al. (2003) have shown in the mouse model that naturally occurring, single, conservative aa substitutions in the HBsAg subtypes can change CD8+ T-cell repertoire to viral antigens. Further studies will be necessary to clarify the implications of the genetic diversity of HBV for T-cell responses.

The anti-HBs MAbs bind to wtHBsAg and mtHBsAg differently. The observed binding of an anti-HBs antibody is a sum of the affinity of the MAb to HBsAg, the concentration of the MAb used in IF staining, the position and the nature of aa substitutions, and many other factors like the experiment procedure. Thus, it is still difficult to compare the data published about the numerous aa

substitutions. It will be desirable to develop a defined panel of antibodies to describe mtHBsAg.

Materials and methods

Construction of plasmids encoding HBsAg with single mutations within the a-determinant by site-directed mutagenesis

A cloned HBV genome of the subtype adw2 pHBV991-12-1 (GenBank accession number X51970) was used as the backbone of the mutated HBV s gene. The cloned DNA was extracted from *E. coli* and subjected to PCR amplification. The region encoding the HBsAg (nt 11–1027 according the numbering of pHBV991-12-1) of HBV was amplified using KS13 (nt 11–36, 5′-CCT TCC ACC AAA CTC TGC AAG ATC CC-3′) and KS14 (nt 1027–1005, 5′-GGA GCA GCA AAG CCC AAA AGA CC-3′) and cloned into pCR2.1 vector (Weinberger et al., 2000). The correctness of the sequence was verified by DNA sequencing analysis of the cloned fragments. Mutations were introduced by PCR-based, site-directed mutagenesis using specific primers. The WT and mutated HBV s genes were recloned into the *Eco*RI site of the expression vector pcDNA3 and tested for the expression of HBsAg by transient transfection in hepG2 cells.

The coding sequence for the HBV preS2 region was added to both mutated s sequences to generate pS2-HBsAg145R and pS2-HBsAg134S using the previously cloned sequence (Zheng et al., 2002). This procedure resulted into plasmids expressing MHBsAg with aa substitutions G145R and Y134S within the S domain.

Two additional expression vectors for immunizations were constructed on the basis of the expression vector E2-TM8, a modified version of pcDNA3 with an EPO leader sequence and a FLAG-tag (Tacke et al., 1997). Two previously characterized clones with known sequences encoding a WT and a mutated HBV s gene of the subtype adw were selected (Seddigh-Tonekaboni et al., 2000). The HBV sequences were reamplified with the primers FS *Not*I 5′-GAG GCG GCC GCC ATG GAG AAC ATC ACA TCA (nt 154–172) GG-3′and RS *Apa*I 5′-CAG GGG CCC CTA TTA AAT GTA TAC CCA GAG AC-3′ (nt 832–816), restricted with *Not*I and *Apa*I, and inserted into E2-TM8 predigested with *Not*I and *Apa*I. The correctness of the cloned sequences was verified by sequence analysis. These procedures resulted into two expression vectors pcDNA3-101 and pcDNA3-146 expressing wtHBsAg and mtHBsAg with triple aa substitutions P142S/G145R/N146D (See below, Fig. 4A). These plasmids were used for genetic immunizations to generate MAbs in mice.

Transient transfection with the expression plasmids

HepG2 cells or baby hamster kidney cells (10^5 per well) were maintained in appropriate media, supplemented with 10% fetal calve serum (FCS) in a eight-well tissue culture chamber slides at 37 °C and 5% CO_2 until the cells reached approximately 50–80% confluence. One to 2 μg of DNA plasmids and 2 μl of LIPOFECTAMINE (GIBCO BRL, Neu-Isenburg, Germany) were diluted with 18 μl of OPTI-MEM, respectively, and then mixed. Following 30–45-min incubation at room temperature, DNA–liposome complexes were diluted in 160 μl of OPTI-MEM and slowly added to prewashed cells. 0.4 ml of fresh RPMI 1640 or MEM containing 10% FCS was added to cells and incubation was continued for 48 h. Supernatants from each well were collected and stored at −20 °C for detection of HBsAg. Transfected cells were washed twice with phosphate-buffered saline (PBS) and fixed with 50% methanol at 4 °C for 30 min for IF staining.

IF staining of transfected cells

Polyclonal goat anti-HBs antibodies to plasma-derived HBsAg of ad and ay types (Biotrend, Köln, Germany. 1:80 dilution with PBS), and monoclonal mouse anti-HBsAg ‘a’ antibodies (Dako, Hamburg, Germany, or Roche, Penzberg, Germany, 1:100 dilution with PBS) were used as primary antibodies for IF staining. One hundred microliters of diluted antibodies were added into each well. After incubation 1 h at 37 °C, cells were washed with PBS three times. Anti-goat-IgG FITC (1:80 dilution) or anti-mouse-IgG FITC (1:100 dilution) were used as secondary antibodies. All of the second antibodies were diluted with PBS containing 0.1% Evans Blue and were incubated at 37 °C for 1 h. Thereafter, cells were washed with PBS and covered with mounting medium for fluorescence microscopy (Zheng et al., 2002).

In addition, IgG fractions were purified from anti-HBs positive human sera by ammonium sulfate precipitation or by protein-G column. Staining of transfected cells were performed with IgG fraction at a dilution up to 1:10 in PBS. FITC labeled anti-human IgG antibodies (Dako) were used as secondary antibody a dilution 1:100.

DNA-based immunization of mice

BALB/cJ (H-2^d) mice were kept under standard-pathogen-free conditions in the Central Animal Laboratory of University Essen. Mice of 6–8 weeks of age were immunized intramuscularly (i.m.) with 100 μg of plasmid DNA as described previously (Lu and Lorentz, 2003; Schirmbeck et al., 1995). The mice received two immunizations in a time interval of 3 weeks and were sacrificed 3 weeks after the last immunization for determination of serum antibody titers to HBsAg, MHBsAg, and mtHBsAg with the aa substitution G145R, and for the detection of HBsAg-specific cytotoxic T-lymphocytes (CTL).

The antibodies to HBsAg in serum samples were detected by the standard commercial assay Enzygnost anti-HBs II kit (Behring, Marburg, Germany) according to

the manufacturer's instructions. The detection of specific antibodies to MHBsAg was performed as described in following protocol: plates were coated with 10 μg of recombinant HBsAg containing large and middle HBsAg per ml in 0.1 M carbonate buffer [pH 9.6] at 37 °C for 1 h and then blocked with 100 μl of 5% FCS for 1 h at 37 °C (the antigens were provided by Dr. J. Reimann; Diminsky et al., 1997). Fifty microliters of mouse serum samples at dilutions 1:10–1:1000 were dispensed into the wells and incubated at 37 °C for 1 h. After washing, 50 μl of horseradish peroxidase-conjugated rabbit anti-mouse-IgG at a dilution 1:1000 was added into each well for 1 h at 37 °C. Fifty microliters of dissolved *O*-phenylenediamine (Sigma-Aldrich Co., Steinheim, Germany) was added as the substrate of horseradish peroxidase into the wells. The development of color occurred at room temperature and was read at 490 nm. The cutoff value was set as three times over negative controls. For detection and titration of antibodies to HBsAg with the aa substitution G145R, mtHBsAg was purified from plasma of a patient and used for ELISA.

For detection of HBsAg-specific CTLs, spleens were taken from sacrificed mice. Splenocytes were cultured with P815 cells expressing HBsAg (kindly provided by Dr. F. V. Chisari) for 5 days. The portion of specific CTLs directed to the dominant H-2L^d-restricted epitope on HBsAg aa 29–38 was determined by the dimer technology (BD Biosciences, CA) according to the manufacturer's instructions. Recombinant soluble dimeric H-2L^d:IgG molecules were loaded by incubation with 460 molar excess of the HBsAg-derived peptides aa 29–38 or aa 208–215 at 4 °C for 48 h. The peptide aa 208–215 is a H-2K^b-restricted CTL epitope and serves as control. The peptides were purchased from EMC microcollections GmbH (Tübingen, Germany) and had a purity >80%. Cultured mouse splenocytes were washed with BD PharMingen staining buffer (BD Biosciences) and concentrated to 5×10^6 per milliliter. Two microliters of peptide-loaded H-2L^d:IgG were added to 200 μl of cultured splenocytes and incubated at 4 °C for 1 h. After three washes, splenocytes were incubated with 2 μl of anti-CD8a FITC (clone 53–6.7) and 2 μl of PE-labeled anti-mouse IgG_1. All antibodies used in these experiments were purchased from BD Biosciences. After staining with labeled antibodies, cells were washed twice, resuspended in 0.5 ml of staining buffer in a tube, and subjected to flow cytometry. Due to the previous in vitro expansion, the above-described method allows only the qualitative detection of specific CTLs. In addition, HBsAg-specific CTLs were detected by Cr-release assay using ^{51}Cr-labeled P815 target cells as described previously (Lu and Lorentz, 2003).

Generation of specific MAbs to mtHBsAg with the mutation G145R

Two expression vectors pcDNA3-101 and pcDNA3-146 were constructed for the genetic immunization of mice. The plasmid pcDNA3-101 expressing the WT adw HBsAg was used as a positive control for the immunization procedure. BALB/cJ (H-2^d) mice were immunized twice with plasmids. One mouse immunized with pcDNA3-146 was further boosted with 800 ng of recombinant mtHBsAg with respective mutations (Weber et al., 2001). Five days after the boost with the recombinant mtHBsAg, hybridoma cells were generated with splenocytes from this mouse and tested for the reactivity to wtHBsAg and mtHBsAg. The selection of the hybridoma cell lines was performed by testing the reactivity of hybridoma supernatants to wtHBsAg and mtHBsAg in ELISA. Two hybridoma cell lines 2.001.2 and 2001.3 were generated to produce MAbs to mtHBsAg with G145R.

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