De Novo Infection in a Renal Transplant Recipient Caused by Novel Mutants of Hepatitis B Virus Despite the Presence of Protective Anti–Hepatitis B Surface Antibody

Mengji Lu and Thomas Lorentz
Institut für Virologie, Universitätsklinikum Essen, Essen, Germany

A renal transplant recipient became persistently positive for hepatitis B virus (HBV) despite preexisting anti–hepatitis B surface (HBs) antibodies. The antigenicity and immunogenicity of wild-type (wt) and mutant HBs antigens (Ags) were compared. In contrast to wt HBsAg, genetic vaccination with mutant HBsAgs did not induce anti-HBs. Thus, mutations within HBsAg may enable HBV to escape immunological control.

Infection with hepatitis B virus (HBV) can be prevented by vaccination with the major HBV surface (HBs) antigen (Ag) [1]. However, HBV isolates carrying mutations within the a-determinant have changed HBs antigenicity and are able to escape immune control and cause infection in vaccinated persons [2, 3]. HBV mutants may also emerge in liver transplant recipients and in infants receiving anti-HBs hyperimmunoglobulin as immunoprophylaxis [4, 5]. Furthermore, HBV mutants are present in chronically HBV-infected patients who do not receive medical treatment, which implies that these mutants circulate naturally and present a source of de novo HBV infection [6, 7]. Therefore, some patients with a compromised immune system, such as transplant recipients, may be at high risk of HBV infection. For example, an HBV mutant with an amino acid substitution at position 133M in the HBsAg caused HBV infection in a liver transplant recipient, even though this patient was vaccinated and had a high titer of anti-HBs [8].

Here, we report that HBV mutants are able to escape immune responses against hepatitis B vaccines and to establish chronic infection in a transplant recipient. These HBV mutants carried multiple amino acid substitutions around and within the HBsAg a-determinant and showed reduced reactivity to a panel of anti-HBs antibodies. Furthermore, we used DNA immunization of mice with plasmids expressing wild-type (wt) and mutant HBsAg to characterize the immunogenicity of wt and mutant HBsAg [9, 10]. We concluded that the antigenicity and immunogenicity of mutant HBsAg were significantly changed and thus led to immunity escape.

Materials and methods. A renal transplant recipient was tested during routine diagnostic surveillance for HBV infection (figure 1). The serological profile (positive for anti-HBs and negative for anti–HBV core antigen [Hbc], anti–HBV e antigen [HBe], HBsAg, and HBeAg) indicated that this patient was successfully immunized against HBV and had not been exposed to HBV infection prior to transplantation. Antibodies to human immunodeficiency virus and hepatitis C virus were not detected. Monitoring was continued routinely after the diagnosis of chronic HBV infection, at intervals of 1 year and, later, of 6 months. HBV DNA was detected by routine diagnostic polymerase chain reaction (PCR) and 

DNA extraction, PCR amplification of the encoding region of HBsAg (nt 157–860), and cloning and sequencing of PCR fragments were done as described elsewhere [10]. The rate of nucleotide misincorporation was \( \sim 10^{-5} \) nt/base/cycle under our PCR conditions, resulting in 1 PCR error/3300 bp.

The HBsAgs sequences containing mutations were recloned into the expression vector pCDNA3 and tested for the expression of HBsAg by transient transfection in baby hamster kidney or HepG2 cells, as described elsewhere [10]. For immunoprecipitation (IP), transfected cells were cultured in medium supplemented with 10% dialyzed fetal calf serum and 20 \( \mu \)g/mL 35S-methionine (10 mCi/mL). Supernatants of transfected cells were collected after 48 h and preabsorbed with 5 mg of protein A–Sephadex (Pharmacia). Five microliters of a cocktail of 6 different anti-HBs monoclonal antibodies (MAbs) was added to the supernatants. After incubation for 30 min at 37\( ^\circ \)C, 5 mg of protein A–Sephadex was added to precipitate HBsAg in complex with anti-HBs antibodies. The HBsAg bound to protein A–Sephadex was dissolved in sample buffer and subjected to
Figure 1.  Hepatitis B virus (HBV) infection of a renal transplant recipient. Antibodies to hepatitis B surface (HBs) antigen (Ag) and hepatitis B core (HBc) Ag and levels of HBsAg and hepatitis B e (HBe) Ag were routinely monitored by ELISAs. HBV DNA was detected by nested polymerase chain reaction (PCR) and quantified by a bDNA assay (Chiron). Negative; +, positive; N, not determined. The time points of the first diagnosis of renal insufficiency (month -36) and transplantation (month 0) were indicated. Eight or 9 clones of PCR fragments derived from samples taken at months 51, 58, and 68 were sequenced. The nos. and types of mutant sequences (K07, K29, K30, and K33) found in each sample are shown. The amino acid variations in the mutant HBsAg are given in table 1.

SDS-PAGE. Immunofluorescence (IF) staining of transfected cells using polyclonal antibodies and MAbs was done as described by Zheng et al. [10]. A commercial HBsAg ELISA assay (Enzygnost HBsAg 5.0; Dade Behring) was used for detection of HBsAg in culture supernatants of transfected cells, according to the manufacturer’s instructions.

Immunization of BALB/cJ (H-2Ld) mice with plasmid DNA was done as described elsewhere [9, 10]. The mice received a total of 3 immunizations 3 weeks apart and were killed 3 weeks after the last immunization. Anti-HBs antibodies in serum samples were detected by use of an Enzygnost anti-HBs II kit (Behring), according to the manufacturer’s instructions. To detect cytotoxic T lymphocyte (CTL) responses to HBsAg, splenocytes of immunized mice were cultured with P815 cells expressing HBsAg (kindly provided by Dr. F. V. Chisari, Scripps Research Institute, La Jolla, CA) for 5 days. HBsAg-specific CTLs were detected by chromium release assay using 51Cr-labeled P815 target cells [9]. The portion of specific CTL responses directed to the H-2Ld-restricted epitope on HBsAg at 29–38 was determined by a novel dimer technology (BD Biosciences), according to the manufacturer’s instructions. Recombinant soluble dimeric H-2Ld IgG molecules were incubated with 460 M excess of the HBsAg-derived peptides at 29–38 or aa 208–215 (EMC Microcollections) at 4°C for 48 h. The control peptide (aa 208–215) is an H-2Kd-restricted CTL epitope. Cultured mouse splenocytes were washed with BD Pharmingen staining buffer and concentrated to 5 × 10^6 splenocytes/mL. Two microliters of peptide-loaded H-2Ld IgG was added to 200 μL of splenocytes and incubated at 4°C for 1 h. After 3 washes, splenocytes were incubated with 2 μL of anti-CD8a fluorescein isothiocyanate (clone 53-6.7) and 2 μL of phycoerythrin-labeled anti–mouse IgG1. 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.

Results. Before renal transplantation (month -36 to month 0), the patient was positive for anti-HBs, with titers of 25–40 U/mL, but was negative for HBsAg, HBeAg, anti-HBc, and anti-HBe (figure 1). HBsAg became undetectable at month 58. Thus, a de novo infection with HBV occurred despite the presence of anti-HBs at a protective level. The source of HBV infection for this patient was not known.

Analysis of the coding region of HBsAg of HBV isolates from this patient at months 51, 58, and 68 after transplantation revealed that heterogeneous HBV populations with different variants were present (figure 1). A mutant K33 was present in all 3 samples analyzed up to month 68 and was predominant by month 68. Two other mutants, K29 and K30, were found in 2 different samples, whereas 1 mutant, K07, was found only
in 1 sample from month 51. All isolates were closely related to the HBV genotype D [11]. These variants carried multiple mutations at amino acid positions 118, 120, 126, 127, 130, 134, 144, and 160 of HBsAg. Mutant types K07, K29, and K30 harbored amino acid substitutions T118R, P120A, P127T, and G130K within the HBsAg a-determinant and K160N, a determinant for HBV subtypes. These 3 mutants differed from each other at amino acid positions 126 (S or I), 130 (N or K), and 144 (D or E). Variations at 7 positions—P120T, G130R, and F137H in particular—were found in mutant type K33 but not in naturally occurring HBV isolates. K33 had a lysine (K) at position 160 of the amino acid sequence, corresponding to the ayw2 subtype [11, 12]. No wt HBV isolate was found among sequenced clones, indicating that these mutants were competent for replication, despite variations in the HBV polymerase sequences (data not shown). The HBV load reached $3.9 \times 10^4$ copies/mL, as determined by the bDNA assay. A population of HBV mutants with multiple amino acid substitutions in the central region of HBsAg established persistent infection.

The expression of wt and mutant HBsAg was examined by transient transfection, labeling with $^{35}$S-methionine, and IP. The expressed HBsAg in supernatants of transfected HepG2 cells was precipitated with a cocktail of 6 different anti-HBs MAbs and analyzed by use of SDS-PAGE. Mutant wt and HBsAgs were detected at a similar level, indicating that the mutations did not affect the expression of HBsAg (table 1). However, the reactivities of wt and mutant HBsAg to single anti-HBs antibodies were different, as shown by indirect IF staining of transiently transfected cells. Cells expressing wt HBsAg and mutant K29 and K33 were positively stained with the polyclonal antibody. Staining of cells expressing HBsAg K30 with the multiple mutations distributed in both loops of HBsAg a-determinant with anti-HBs polyclonal antibody was negative. An MAb with a high affinity (number 2 in table 1) recognized wt and all 3 HBsAg variants equally. Mutant HBsAg K29 and K33 were positively stained by 2 and 3 MAbs, respectively, which is consistent with the fact that they had no amino acid substitution within the second loop of the HBsAg a-determinant (aa 139–147). The other 3 MAbs did not recognize K30. Furthermore, wt HBsAg was detected in ELISAs at high levels in supernatants of transfected cells, whereas only a weak signal for HBsAg was detected in supernatants of cells transfected with all 3 different plasmids expressing mutant HBsAg (data not shown).

Vaccination with plasmids encoding recombinant viral proteins provides a useful method of analyzing B and T cell epitopes on hepatitis B virus surface antigens [10]. Three intramuscular injections of 100 μg of DNA plasmids expressing wt HBsAg induced anti-HBs titers of 68–2500 IU/mL in 4 of 5 mice (table 1). Only 1 of 4 mice became anti-HBs positive, with a titer of 460 IU/mL, after immunization with plasmid-expressing mutant HBsAg K29. No anti-HBs antibody was detected in mice after immunization with mutant HBsAg K30.

Table 1. Properties of mutant hepatitis B surface (HBs) antigen (Ag) identified in a renal transplant recipient.

<table>
<thead>
<tr>
<th>HBsAg type</th>
<th>Amino acid substitutions at relevant positions</th>
<th>Anti-HBs level, IU/mL</th>
<th>Immune responses in mice after 3 immunizations with plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>ayw</td>
<td>adw</td>
</tr>
<tr>
<td></td>
<td></td>
<td>118</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>F</td>
</tr>
<tr>
<td>wt</td>
<td></td>
<td>R</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>K29</td>
<td>R</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>K30</td>
<td>R</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>K33</td>
<td>T</td>
<td>T</td>
</tr>
</tbody>
</table>

**NOTE.** CTL, cytotoxic T lymphocyte; IF, immunofluorescence; IP, immunoprecipitation; MAb, monoclonal antibody.

|            |   | 118 | 120 | 126 | 130 | 134 | 144 | 150 | 160 | 168 |   |   |   |   |
|            |   | T   | P   | G   | D   | G   | K   | A   | +   | ++ | ++ | ++ | ++ | ++ | 2500, 2400, 1500, 68, <9 | 31.0, 49.9 | 34.1, 28.7 |
|            |   | F   | A   | V   | A   |   |   |   |   |   |   |   |   |   |   |
|            |   | I   | F   | A   | R   | V   |   |   |   |   |   |   |   |   |   |
| wt         |   | R   | A   | S   | T   | N   | Y   | D   | G   | N   | A   |   |   |   |   |
|            | K29 | R   | A   | I   | T   | K   | Y   | D   | G   | N   | A   | +   | ++ | +   | -- | -- | -- | -- | 460, <9, <9, <9 | 37.6, 42.7 | 25.8, 36.6 |
|            | K30 | R   | A   | I   | T   | K   | Y   | E   | G   | N   | A   | +   | ++ | -- | -- | -- | -- | <9, <9, <9, <9, <9, <9 | 45.5, 41.1 | 60.1, 40.5 |
|            | K33 | T   | T   | T   | P   | R   | H   | D   | G   | K   | A   | +   | ++ | +   | ++ | +   | -- | <9, <9, <9, <9, <9, <9, <9 | 32.4, 40.8 | 46.3, 14.2 |

a Only the amino acid residues at the relevant positions of 3 subtypes ayw, adw, and ayr and 4 mutant HBsAgs from this study are given, according to Norder et al. [11].

b IP of $^{35}$S-methionin–labeled HBsAg in supernatants of transfected cells was carried out with a cocktail of 6 anti-HBs MAbs. +, Detection of HBsAg.

c IF staining of transfected cells was performed with anti-HBs polyclonal antibody (no. 1) and different anti-HBs MAbs (nos. 2–5). ++, Normal staining; +, positive staining with reduced intensity; --, negative.

d The anti-HBs titers in each serum sample from mice after 3 immunizations are given separately.

e The anti-HBs titers in each group were determined by chromium release assay. The specific lysis of target P815 cells expressing middle HBsAg was measured with an effector-to-target ratio of 10:1 in a chromium release assay (see “Materials and methods”). The spontaneous release rate was <10%. The unspecific lysis of P815 control cells by effector cells was <11.3%.

f Cultured HBsAg–specific CTLs from 2 mice from each group were stained with specific dimer to HBsAg aa 29–38, as described in “Materials and methods.” The percentage of dimer-positive CD8+ cells is given. The CD8+ cells were 43%–75% of total cell counts. The unspecific staining of CD8+ cells with control dimers is <3%.
and K33. These results indicate that mutant HBsAg had a reduced ability to induce anti-HBs antibody in mice.

The amino acid substitutions found in mutant HBsAg are not supposed to affect the induction of HBsAg-specific CTL responses. Consistently, immunized mice developed CTL responses to HBsAg without an obvious difference between these different groups, as tested in chromium release assay (table 1). Furthermore, the CTL responses to the immunodominant, H-2L^d-restricted epitope on HBsAg aa 29–38 was analyzed for each expanded culture. Approximately 70% of cells in cultures after 7 days were positively stained with anti-CD8 (data not shown). Twenty-six percent to 60% of cells were double-stained with anti-CD8 and H-2L^d IgG dimeric protein loaded with HBsAg (aa 29–38; table 1), whereas dimeric proteins loaded with a control peptide (aa 208–215) stained <3% of cells. Mice immunized with control plasmids, such as pcDNA3, did not develop any HBsAg-specific CTL responses (data not shown). Thus, DNA immunization using plasmids expressing mutant HBsAg primed a normal HBsAg-specific CTL response, although the induction of anti-HBs antibody response was impaired.

Discussion. In the present study, we documented an unusual case of de novo HBV infection in an adult that occurred despite preexisting protective antibody. Our data indicate that a persistent HBV infection was established with a heterogeneous population of HBV variants carrying several novel mutations within the HBsAg a-determinant [13]. In particular, the K160N substitution has been found once by examination of a sample of subtype ad, which is devoid of the w or r determinant [14]. The selection of the mutation at aa 160 of HBsAg suggests that subtype W-specific antibodies to HBsAg may be able to neutralize HBV.

DNA immunization was found to be a useful tool for assessing the immunogenicity of mutated HBsAg. We demonstrated that the immunogenicity of mutant HBsAg was significantly impaired, even though their antigenicity was only partly reduced. Mutant HBsAg did not stimulate an increased production of anti-HBs in the patient we studied.

The immunological control of HBV is achieved by both B and T cell responses [15]. Immunosuppressive treatment after organ transplantation apparently leads to an impairment of T cell functions and allows HBV mutants to escape the protective antibody responses. Thus, transplant recipients may be at risk for infection with HBV mutants despite prior vaccination.

Acknowledgments

We thank Thekla Kemper and Barbara Bleekmann for excellent technical assistance, Masanori Isogawa for support, M. Roggendorf for continuous support and helpful discussion, and W. H. Gerlich and J. Waters for critical reading of this manuscript and helpful comments.

References