Coexistence of Hepatitis B Surface Antigen (HBsAg) and Heterologous Subtype-Specific Antibodies to HBsAg among Patients with Chronic Hepatitis B Virus Infection

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(See the editorial commentary by Gerlich on pages 1170–2)

Background. The coexistence of hepatitis B surface antigen (HBsAg) and antibodies to HBsAg (anti-HBs) in patients with chronic hepatitis B virus (HBV) infection has been explained by the presence of viral escape mutants. Yet, no systematic analysis of such patients has been performed. We analyzed both the HBV strains and the nature of anti-HBs in such patients.

Methods. Four hundred eleven patients with chronic HBV infection were tested for the presence of anti-HBs. The sequences of the HBsAg coding region were analyzed. Anti-HBs were purified and examined in commercial assays alone and with 3 different HBsAg subtypes.

Results. Twenty patients had positive results for anti-HBs. This serological status remained stable for 12 months (as tested thus far). Amino acid substitutions and/or variations on HBsAg were found in 13 patients, and the HBV isolates from 4 others were wild types. Importantly, no significant difference in the occurrence of amino acid substitutions within the HBsAg was found in HBV isolates from patients with and without anti-HBs. Purified immunoglobulin fractions from serum samples from patients were reactive to HBsAg but had a lower specific activity, compared with those taken from immunized persons. Anti-HBs in patients were directed to the HBsAg subtypes other than the coexisting one. No circulating immune complex could be detected in these patients.

Conclusion. HBsAg and anti-HBs with an unmatched specificity coexisted in 4.9% of patients. The presence of anti-HBs was not associated with the appearance of specific HBV mutants in patients with chronic infection. Apparently, the presence of anti-HBs in patients with chronic HBV infection did not lead to a selection of HBV escape mutants.

Hepatitis B virus (HBV) causes acute, self-limiting, and chronic infections in humans [1]. The antibody response to hepatitis B surface antigen (HBsAg) appears during the late phase of infection. The antibodies to HBsAg (anti-HBs) are able to neutralize HBV infectivity and, therefore, clear circulating HBsAg and infectious HBV particles from peripheral blood. The presence of anti-HBs is considered an indicator of immunity to HBV infection. Vaccination with HBsAg is proven to be effective to prevent HBV infection [2].

The typical serological feature of chronic HBV infection is the presence of circulating HBsAg in patients and lack of anti-HBs [1]. However, the coexistence of HBsAg and anti-HBs in patients with chronic HBV infection has been often reported [3–13]. Early reports indicated that anti-HBs in such patients may be specifically directed to HBsAg subtypes different from the coexisting HBsAg [3–7]. Because of technical limitations, no sequence analysis of HBV isolates in patients was performed in these early studies. Later reports suggested that the presence of anti-HBs may drive the selection of HBsAg escape mutants [13]. It has been shown that amino acid substitutions may occur in the
Table 1. Comparison of demographic, clinical, and virologic characteristics of patients with chronic hepatitis B virus between the antibodies to hepatitis B surface antigen (anti-HBs)–positive and –negative group.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Serum anti-HBs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (n = 20)</td>
</tr>
<tr>
<td>Age, median years (range)</td>
<td>38 (21–52)</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>8/12</td>
</tr>
<tr>
<td>Serum ALT level, median IU/L (range)</td>
<td>139 (89–570)</td>
</tr>
<tr>
<td>Serum HBV DNA level, mean log 10 copies/mL/SD</td>
<td>6.3 ± 0.21</td>
</tr>
<tr>
<td>HBeAg titer, mean S/N ± SD</td>
<td>144.8 ± 22.4</td>
</tr>
<tr>
<td>HBsAg titer, mean S/N ± SD</td>
<td>322.6 ± 20.0</td>
</tr>
</tbody>
</table>

**NOTE.** ALT, alanine aminotransferase; HBV, hepatitis B virus; HBeAg, hepatitis B early antigen; HBsAg, hepatitis B surface antigen; S/N, signal-to-noise ratio.

a-determinant of HBsAg of HBV isolates and reduce or abolish the binding of anti-HBs to HBsAg, leading to immune escape [8–13]. However, HBV isolates from patients with HBsAg and anti-HBs were often without relevant mutations in the coding region of HBsAg. Frequently, mutant HBV isolates were accompanied by wild-type isolates, forming a mixed viral population in patients [8, 10, 11]. These facts contradict the hypothesis that HBV escape mutants are selected by coexisting anti-HBs.

In our study, we examined both the HBsAg sequences and the nature of anti-HBs in patients with chronic HBV infection and determined the relationship of the HBsAg sequence variation and the specificity of anti-HBs. Our results indicated clearly that the presence of anti-HBs was not correlated with

Table 2. Summary of clinical characteristics of patients with both hepatitis B surface antigen (HBsAg) and antibodies to hepatitis B surface antigen (anti-HBs).

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Age, years</th>
<th>Sex</th>
<th>HBsAg, S/N</th>
<th>Anti-HBs, a mIU/mL</th>
<th>HBV-DNA</th>
<th>Family history of HBV infection</th>
<th>ALT level, IU/L</th>
<th>Genotype/ HBsAg subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>LWC01</td>
<td>31</td>
<td>M</td>
<td>452.75</td>
<td>&gt;1000</td>
<td>1.12E+05</td>
<td>No</td>
<td>101</td>
<td>B2/adw2</td>
</tr>
<tr>
<td>WXG02</td>
<td>34</td>
<td>M</td>
<td>451.42</td>
<td>12.6</td>
<td>1.55E+06</td>
<td>No</td>
<td>135</td>
<td>B2/adw2</td>
</tr>
<tr>
<td>SWJ03</td>
<td>31</td>
<td>F</td>
<td>605.84</td>
<td>32.9</td>
<td>1.25E+06</td>
<td>No</td>
<td>187</td>
<td>B2/adw2</td>
</tr>
<tr>
<td>YQY04</td>
<td>29</td>
<td>F</td>
<td>221.31</td>
<td>12.1</td>
<td>3.97E+06</td>
<td>No</td>
<td>144</td>
<td>B2/adw2</td>
</tr>
<tr>
<td>YPF05</td>
<td>31</td>
<td>F</td>
<td>370.52</td>
<td>&gt;1000</td>
<td>1.94E+08</td>
<td>No</td>
<td>101</td>
<td>C1/adw2</td>
</tr>
<tr>
<td>HJ006</td>
<td>25</td>
<td>M</td>
<td>290.37</td>
<td>169</td>
<td>1.58E+05</td>
<td>No</td>
<td>118</td>
<td>C1/adrq+</td>
</tr>
<tr>
<td>JJ007</td>
<td>29</td>
<td>F</td>
<td>257.31</td>
<td>975.4</td>
<td>3.19E+05</td>
<td>No</td>
<td>151</td>
<td>C1/adrq+</td>
</tr>
<tr>
<td>YMF08</td>
<td>26</td>
<td>F</td>
<td>324.94</td>
<td>19.7</td>
<td>3.73E+05</td>
<td>No</td>
<td>121</td>
<td>C1/adrq+</td>
</tr>
<tr>
<td>JJW09</td>
<td>35</td>
<td>M</td>
<td>318.46</td>
<td>35</td>
<td>6.74E+05</td>
<td>Yes</td>
<td>198</td>
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</tr>
<tr>
<td>WHU10</td>
<td>28</td>
<td>M</td>
<td>269.35</td>
<td>21.7</td>
<td>1.03E+06</td>
<td>Yes</td>
<td>211</td>
<td>C1/adrq+</td>
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<tr>
<td>LWF11</td>
<td>28</td>
<td>M</td>
<td>273.38</td>
<td>10.7</td>
<td>3.21E+06</td>
<td>Yes</td>
<td>89</td>
<td>C1/adrq+</td>
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<tr>
<td>LF012</td>
<td>39</td>
<td>F</td>
<td>330.4</td>
<td>11.7</td>
<td>4.19E+06</td>
<td>No</td>
<td>149</td>
<td>C1/adrq+</td>
</tr>
<tr>
<td>WXG13</td>
<td>35</td>
<td>M</td>
<td>256.69</td>
<td>471.4</td>
<td>5.75E+06</td>
<td>No</td>
<td>165</td>
<td>C1/adrq+</td>
</tr>
<tr>
<td>WZM14</td>
<td>35</td>
<td>M</td>
<td>233.27</td>
<td>157.9</td>
<td>5.49E+06</td>
<td>Yes</td>
<td>223</td>
<td>C1/adrq+</td>
</tr>
<tr>
<td>ZYJ15</td>
<td>41</td>
<td>M</td>
<td>283.86</td>
<td>149.12</td>
<td>7.10E+06</td>
<td>No</td>
<td>109</td>
<td>C1/adrq+</td>
</tr>
<tr>
<td>LFF16</td>
<td>27</td>
<td>F</td>
<td>418.83</td>
<td>108.3</td>
<td>9.18E+06</td>
<td>No</td>
<td>109</td>
<td>C1/adrq+</td>
</tr>
<tr>
<td>CZY17</td>
<td>22</td>
<td>M</td>
<td>327.92</td>
<td>664.5</td>
<td>2.30E+07</td>
<td>Yes</td>
<td>258</td>
<td>C1/adrq+</td>
</tr>
<tr>
<td>XZT18 b</td>
<td>32</td>
<td>M</td>
<td>340.2</td>
<td>389.2</td>
<td>5.60E+07</td>
<td>Yes</td>
<td>570</td>
<td>C1/adrq+</td>
</tr>
<tr>
<td>JXJ19</td>
<td>32</td>
<td>M</td>
<td>309.84</td>
<td>51.5</td>
<td>1.33E+08</td>
<td>No</td>
<td>97</td>
<td>C1/adrq+</td>
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<tr>
<td>ZOH20</td>
<td>27</td>
<td>M</td>
<td>298.58</td>
<td>254.4</td>
<td>4.22E+08</td>
<td>Yes</td>
<td>109</td>
<td>C1/adrq+</td>
</tr>
</tbody>
</table>

**NOTE.** All patients were positive for antibodies to hepatitis B virus core antigen (anti-HBc) and did not receive a specific vaccine against hepatitis B virus (HBV). ALT, alanine aminotransferase; S/N, signal-to-noise ratio.

a Anti-HBs was detected by 1 kit (Abbott Laboratories) twice and redetected by 2 other commercial kits.

b The only patient with cirrhosis.
Anti-HBs among Patients with Chronic HBV

MATERIALS AND METHODS

Patients. From July to October 2003, 411 consecutive Chinese patients with chronic hepatitis B from Shanghai, Beijing, Guangzhou, and Changchun were included in our study. Diagnosis of clinical chronic hepatitis B was based on positive results for HBsAg for at least 6 months, increased serum alanine aminotransferase activity (at least 2 times the upper limit of normal), and positive results for hepatitis B early antigen (HBeAg), along with associated symptoms. Exclusion criteria included decompensated liver disease, previous treatment for chronic hepatitis B, and coinfection with hepatitis C virus, hepatitis D virus, or HIV. All patients provided written, informed consent prior to entrance into this study.

Serologic testing. Serum samples were collected from patients and stored at ~70°C. Serological markers for HBV infection were determined using commercially available EIA kits (Abbott Laboratories) and were confirmed partly with other assays (Roche Diagnostics and Dade Behring). Serum HBV DNA level was quantified using a commercially available real-time fluorescence quantitative kit (Da An Gene Diagnostic Center). This kit was approved by the State Food and Drug Administration (China) with a detection limit of 500 HBV copies/mL. For assay calibration, the standard serum HBV DNA level, supplied by the National Institute for the Control of Pharmaceutical and Biological Products (China), was used.

DNA extraction from serum samples, PCR amplification, and cloning and sequencing of PCR fragments. DNA was extracted from serum samples from patients, using QIAamp DNA blood mini kit (Qiagen), and was subjected to PCR amplification using the high-fidelity Taq polymerase (Roche) in accordance with the manufacturer’s instructions. The region encoding the HBsAg (nt 2818–888) was amplified using the primers FS-S1 5′-GTCACCATATTCTTGGAAC-3′ and S7D 5′-GTTCAGGAAAATATTTGCT-GGGACCCTG-3′ (nt 131–148) and S7D 5′-GACACCAAGCTTGTAGGTTTAAATGTACCTAC-3′ (nt 845–825).

Detection of subtype-specific anti-HBs. Patient serum samples were incubated with recombinant HBsAg specimens of the subtypes adw (genotype A), adr (genotype B), and ayw (genotype D) at a concentration of 10 µg/mL for 30 min and then tested in a commercial anti-HBs assay (Abbott Laboratories).

RESULTS

Patients tested positive for both HBsAg and anti-HBs. Twenty of 411 patients with chronic HBV infection had positive results for anti-HBs in 3 different commercial ELISAs. In addition, 3 patients had positive results for anti-HBs only with the Abbott assay and were not further considered in the analysis. Table 1 shows the clinical characteristics of the patient groups. There was no significant difference between patients with and without anti-HBs with regard to age, sex, serum alanine aminotransferase level, serum HBeAg level, and serum HBsAg level. On average, the patients with anti-HBs had lower serum HBV DNA concentrations than did the patients without detectable anti-HBs (P = .0127).

Serial serum samples from 3 patients (JJ007, YPF05, and WZM14) were collected over a period of 12 months. All 3 patients had positive results for HBsAg and anti-HBs during this period (figure 1; online only), indicating that such a se-

Figure 1 (online only). Dynamic changes of serial antibodies to hepatitis B surface antigen (anti-HBs; ▲), hepatitis B surface antigen (HBsAg; ●), alanine aminotransferase (ALT) level (■), and serum hepatitis B virus (HBV) DNA level (▲) in serum samples from 3 representative patients at 1-year follow-up. A and B, Patient JJ007. C and D, Patient YPF05. E and F, Patient WZM14.

with 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. The rate of nucleotide misincorporation was ~10⁻⁸ base pairs/cycle under our PCR condition, resulting in ~1 PCR error per 3300 base pairs. Sequence analysis was performed directly with PCR products or with cloned DNA, according to Ireland et al. [14], using the primers 6DC 5′-GCACACCGATTCGAGGACTG-GGGACCCTG-3′ (nt 131–148) and 7DC 5′-GACACCAAGCT-TGTAGGTTTAAATGTACCTAC-3′ (nt 845–825).

Purification of IgG fractions from human serum samples. IgG fractions were purified from serum samples from patients by ammonium sulfate precipitation [15] or by ImmunoPure (G) IgG purification kit (Pierce), according to the manufacturer’s instruction. The amounts of purified IgGs were determined by Biorad protein assay (Biorad). The reactivity of purified anti-HBs to HBsAg was determined by ELISA.

Figure 2 (online only). The relations of antibodies to hepatitis B surface antigen (anti-HBs), hepatitis B surface antigen (HBsAg), and serum hepatitis B virus (HBV) DNA level in patients with chronic HBV infection who had positive results for anti-HBs. A, The serum anti-HBs titers measured using diagnostic assays (Roche and Abbott) and compared in the plot. B, Serum HBsAg levels and anti-HBs titers determined by the Abbott assay. C, Serum HBsAg levels and HBV DNA concentrations. D, Serum anti-HBs titers measured by the Abbott assay and serum HBV DNA concentrations.

The figure is available in its entirety in the online edition of Clinical Infectious Diseases.
Figure 3. Amino acid substitutions and variations in hepatitis B surface antigen (HBsAg) in chronic hepatitis B patients with and without antibodies to HBsAg (anti-HBs). Only such positions in HBsAg are indicated if amino acid substitutions were present. The differences of the hepatitis B virus (HBV) subtypes B and C (wild-type sequence [wt] B and wt C) are indicated. The part within the HBsAg a-determinant is underlined. The patients were sorted according to the presence of amino acid substitutions in the HBsAg a-determinant and outside of the HBsAg a-determinant or in both regions. Patient YPF05 with amino acid residue K may be regarded as a mutant but is not listed in the figure. Pos, the amino acid positions in HBsAg.

HBsAg levels varied from 221.31 to 605.84 (signal-to-noise ratio) in this patient group (figure 2A; online only). The blots of serum HBsAg titers and serum HBV DNA concentrations against anti-HBs titers did not indicate a correlation between these parameters among these patients (figure 2C and 2D; online only).

Sequence analysis of HBV isolates in patients with and without anti-HBs. The occurrence of HBV mutants with amino acid substitutions on HBsAg may be a possible expla-
nation for the coexistence of anti-HBs and HBsAg. Thus, we analyzed the sequence encoding HBsAg of HBV isolates from patients with and without anti-HBs by PCR amplification and sequencing. The results are summarized in figure 3 and figure 4D (online only). The analyzed HBV isolates from patients with anti-HBs belonged to the HBV genotypes B2 and C1 [16]. Four HBV isolates of the subtype B2 were classified to the HBsAg subtype adw2 according to the deduced amino acid sequence. Fifteen HBV isolates of the subtype C were of the HBsAg subtype adq+, and 1 belonged to the HBsAg subtype adw2. The amino acid substitutions within the HBsAg sequence are shown in figure 3. Four patients had no differences in the consensus sequences of the HBsAg of the corresponding genotypes. Thirteen patients had single or multiple amino acid substitutions and/or variations within and/or outside of the HBsAg a-determinant. The amino acid position 126 revealed amino acid variations Ile to Thr or Ser that frequently occurred in patients with chronic HBV infection. Other HBV isolates had amino acid substitutions Q129R, G130N, M133T, and T143M within the a-determinant, which was similar to findings in previous studies [9, 17–20].

Four to 7 clones were generated and sequenced for samples from 4 representative patients (YPF05, WXG13, JJ007, and WZM14) (figure 4D; online only). Each of clones from these patients had differences at various positions to the corresponding sequence derived from direct sequencing analysis. These differences in the sequences were present in patient samples, because PCR artifacts would generate only 1 mutation in 5 clones. For example, all 4 HBsAg clones from WZM14 had the amino acid substitutions and/or variations T5A, Q129R, and G130N. The amino acid substitution T47K was found in 3 of 4 clones. Furthermore, 2 clones (WZM-C2 and -C5) had additional amino acid substitutions (L175S, L215I, and F219L). These data confirmed that HBV populations in patients are heterogeneous.

The HBsAg sequences from 26 patients with chronic HBV infection without anti-HBs were analyzed (figure 3). Fourteen patients were infected with HBV of the genotype B2/HBsAg subtype adw2. The HBV isolates in 12 patients belonged to the genotype C1/HBsAg subtype adq+. A total of 12 amino acid substitutions and/or variations were found within the HBsAg a-determinant. A number of additional amino acid substitutions and/or variations were found outside of the a-determinant. Approximately one-half of the amino acid substitutions occurred at amino acid position 126 of HBsAg. Therefore, the

Figure 5. The reactivity of antibodies to hepatitis B surface antigen (anti-HBs) in serum and purified IgG fractions from vaccinated persons and patients with chronic hepatitis B virus (HBV) infection with anti-HBs. IgG fractions were purified from serum samples from 15 vaccinated persons (1–15), 1 anti-HBs-negative control subject (16), and 12 patients with chronic HBV infection with anti-HBs. Serum and purified IgG fractions were tested for the reactivity to hepatitis B surface antigen (HBsAg) in anti-HBs ELISA. A, Anti-HBs titers for serum samples from vaccinated persons and the control subject. B, Anti-HBs titers for serum samples from patients with HBsAg and anti-HBs. C, Anti-HBs titers for IgG fractions from serum samples from vaccinated persons and the control subject. D, Anti-HBs titers for IgG fractions from serum samples from patients with HBsAg and anti-HBs. E, The ratios of anti-HBs titers in IgG fractions and corresponding serum samples presented as percentages for vaccinated persons and the control subject. F, The ratios of anti-HBs titers in IgG fractions and corresponding serum samples presented as percentages for patients with HBsAg and anti-HBs.
Figure 6. The reactivity of positive serum antibodies to hepatitis B surface antigen (anti-HBs) in an anti-HBs immunoassay after preincubation with specimens of recombinant hepatitis B surface antigen (HBsAg) subtypes adw, adr, and ayw. Serum samples from patients were diluted in PBS (1:20) and incubated with recombinant HBsAg at a concentration of 10 μg/mL for 30 min and tested, subsequently, in an anti-HBs assay (Abbott Laboratories).

The frequencies of amino acid substitutions and/or variations were comparable in patients with and without anti-HBs in the HBsAg sequences.

**Anti-HBs in patients.** Another explanation for the coexistence of anti-HBs and HBsAg could be that the ability of binding of anti-HBs to HBsAg is low. Therefore, we characterized anti-HBs from these patients and from 15 persons immunized with HBsAg for comparison. A subject with a negative serum sample was included as a control subject.

Modified anti-HBs ELISA tests using human IgG- and IgM-specific antibodies revealed that both HBsAg-specific IgG and IgM were present in patient serum samples, as well as in serum samples from vaccinated persons (data not shown). IgG fractions from patient serum samples were purified and tested for the binding of anti-HBs to HBsAg. The results are summarized in figure 5. Purified IgGs from vaccinated persons were strongly reactive to HBsAg in ELISA (figure 5A and 5C). All purified IgG preparations were adjusted to a concentration of 5 mg/mL and tested in a commercial anti-HBs ELISA. Six samples from vaccinated persons 1–6 had anti-HBs titers >2000 mIU/mL. The diluted IgG preparations of 5 mg/mL from these samples had anti-HBs titers ranging from 600 to 5120 mIU/mL. Serum
samples from vaccinated persons 7–15 had lower anti-HBs titers (50–944 mIU/mL). Correspondingly, the IgG preparations adjusted to 5 mg/mL showed anti-HBs titers of 13–375 mIU/mL. In general, the reactivity of the IgG preparations at 5 mg/mL corresponded to 13%–63% of the anti-HBs titers of the serum samples (figure 5E). An exceptionally high anti-HBs titer of 5120 mIU/mL was measured in the IgG preparation from 5 serum samples.

Twelve serum samples from patients with positive results for both HBsAg and anti-HBs were available for purification of IgG fractions. The IgG preparations from patients with positive results for both HBsAg and anti-HBs had very low reactivity at a concentration of 5 mg/mL (figure 5B and 5D). The reactivity of the IgG preparation from 6 serum samples was below the cutoff (9 mIU/mL). Particularly, the IgG preparation from many serum samples with high titers >1000 IU/L (from patients WXG13, XZT18, and HJ006) had low reactivity of 6.7, 27, and 30.9 mIU/mL, respectively. Only the IgG of patient YPF05 had an anti-HBs reactivity of 128.4 mIU/mL. The reactivity of the IgG preparations at 5 mg/mL corresponded to 2%–18% of the anti-HBs titers of the serum samples (figure 5F). The only exception, patient JXJ19, had an anti-HBs titer of 21.5 mIU/mL in the IgG concentration 5 mg/mL. Thus, the IgG anti-HBs in patients with HBsAg and anti-HBs were reactive to HBsAg but with a lower reactivity, compared with those from vaccinated persons.

**The specificity of anti-HBs in patient serum samples to HBsAg subtypes.** It has been reported that anti-HBs in patients with chronic HBV infection may be selectively directed to HBsAg subtypes. To detect the subtype-specific anti-HBs, patient serum samples in appropriate dilutions (1:1 to 1:50) were preincubated with recombinant HBsAg specimens of the subtypes adw (genotype A), adr (genotype C), and ayw (genotype D). Normal anti-HBs–positive serum samples from vaccinated persons became negative in the anti-HBs immunoassays after preincubation with specimens of each of 3 recombinant subtypes of HBsAg (figure 6). Approximately 5 g of HBsAg were sufficient to completely absorb anti-HBs with a titer >400 mIU/mL. For serum samples from patients LWCO1 and YPF05, who were infected with the HBV subtype adw, preincubation with specimens of HBsAg of the subtypes adw and adr did not change the anti-HBs titers. Preincubation with specimens of HBsAg subtype ayw led to a complete neutralization of anti-HBs reactivity in these serum samples, indicating that the anti-HBs in these patients were directed to HBsAg subtype determinant y. Six patients (HJ006, JJ007, WZM14, ZYJ15, XZT18, and ZQH20) were infected with the HBV subtype adr. Preincubation with specimens of HBsAg subtype adr did not reduce the anti-HBs reactivity of the serum samples, but anti-HBs reactivity was reduced after preincubation with specimens of HBsAg subtype adw. The remaining 2 serum samples (from patients HJ006 and ZYJ15) became anti-HBs negative in ELISA after a preincubation with specimens of HBsAg subtype ayw. Therefore, all samples tested thus far contained a specific anti-HBs directed to an HBsAg subtype determinant.

**The binding of anti-HBs to HBsAg in serum.** Circulating immune complexes consisting of HBsAg and anti-HBs were reported to exist in HBV-infected patients [21–24]. Possibly, part of specific anti-HBs may be engaged in circulating immune complexes and, therefore, may not be detectable in ELISAs. Thus, we tested whether HBsAg and anti-HBs formed complexes by depletion of serum IgGs that would lead to the concomitant removal of HBsAgs bound to IgG anti-HBs. Figure 7 shows an example of these experiments. Patient YPF05 had a high titer of anti-HBs (>1000 mIU/mL) and HBsAg wild types (∼370; S/N ratio) over 12 months. One-tenth to 2 μL of serum was incubated with 50 μL of protein G–sepharose suspension (Amersham-Pharmacia Biotec) for 15 min at room temperature, resulting in the removal of >90% of IgGs (as tested in ELISA). The incubation supernatants were collected and tested for HBsAg in standard ELISAs (figure 7). The depletion of IgGs did not lead to a significant reduction of HBsAg concentrations. For control, this same procedure was able to remove HBsAg specimens from serum samples if 1 μL of monoclonal anti-HBs was added to serum samples from the patients. Similar results were obtained with serum samples from other patients with lower anti-HBs titers. These results clearly demonstrated that anti-HBs in sera of such patients did not sig-
significantly contribute to the removal of HBsAgs from peripheral blood.

DISCUSSION

In the present study, we examined patients with chronic HBV infection with both HBsAgs and anti-HBs circulating in peripheral blood. Our findings indicated that HBV persistence in the presence of anti-HBs is not associated with emergence of HBV escape mutants with changed HBsAg sequences. Similar numbers of amino acid substitutions were found in the HBsAg sequences derived from patients with and without anti-HBs. The majority of the substitutions represented natural sequence variations and were presumably not related to immune escape. Some amino acid substitutions, such as Q129R and I126S, have been identified previously in cases of potential immune escape. The mutation Q129L was found in an HBV isolate from a vaccinated person, and it reduced binding of anti-HBs [18, 19]. In contrast, mutation I126S occurred frequently in HBV isolates from patients with chronic HBV infection and may represent a polymorphism among the HBV genotype C isolates [8–12, 20], although without a known biological significance. Additional investigations on these genetic variations are needed to clarify their biological significance for viral persistence. Overall, our results do not support the hypothesis made by Lada et al. [13] that the presence of anti-HBs in patients with chronic HBV-infection would lead to the selection of HBV escape mutants. Many patients included in the study by Lada et al. [13] had received various medical interventions. It is not yet clear, but it is likely that antiviral treatments may exert selective pressure on HBsAg and, therefore, increase the rate of amino acid substitutions.

The anti-HBs present in our patients tested thus far were specific against HBsAg subtype determinants. It is not completely excluded that a small fraction of anti-HBs with homologous specificity existed in complexes with HBsAg. Our results indicate that none or only a few circulating immune complexes of anti-HBs and HBsAg were formed in patient serum. Thus, the major fraction of anti-HBs in such patients had the “wrong” specificity and appeared to be unable to bind to serum HBsAg in the same patient. Logically, the presence of such anti-HBs would not lead to a selection of HBV mutants. Consistently, the HBsAg sequence from several patients in this study had the wild-type sequences of HBsAg. The cloning and expression of HBsAg from one of these patients clearly confirmed this fact. Thus, not the mutated HBsAg, but rather, the nature of anti-HBs explains the coexistence of HBsAg and anti-HBs in such patients.

A screening of 411 patients with chronic HBV infection from different regions in China revealed that a relatively high percentage (4.9%) of these persons had detectable serum anti-HBs levels. It is not clear how subtype-specific anti-HBs were induced in patients with chronic infection. Tabor et al. [25] were able to induce the anti-HBs to the “y” determinant of HBsAg in a chimpanzee carrier of HBsAg subtype “adw.” Similarly, therapeutic vaccinations with HBsAg subtype adw could induce subtype-specific anti-HBs in patients (authors’ unpublished data). Immunizations in a woodchuck model led to similar results [26]. Thus, the exposure of patients to HBV infection of different subtypes could not be excluded as a possible explanation for our observation.

Nevertheless, the presence of anti-HBs in patients with chronic HBV infection suggests that these patients were able to mount HBV-specific immune responses. It will be interesting to follow up on these patients to clarify whether the ability to mount HBV-specific immune responses would positively influence the clinical course and act synergistically with antiviral agents.

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References

10. Mesenas SJ, Chow WC, Zhao Y, Lim GK, Oon CJ, Ng HS. Wild-type and “a” epitope variants in chronic hepatitis B virus carriers positive


