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Cellular Immune Responses to the Hepatitis B Virus Polymerase

Eishiro Mizukoshi,3* John Sidney,† Brian Livingston,‡ Marc Ghany,* Jay H. Hoofnagle,∗ Alessandro Sette,† and Barbara Rehermann4∗

CD4⁺ T cells play an important role in hepatitis B virus (HBV) infection by secretion of Th1 cytokines that down-regulate HBV replication, and by promoting CD8⁺ T cell and B cell responses. We have identified and characterized 10 CD4⁺ T cell epitopes within polymerase and used them to analyze the immunological effects of long-term antiviral therapy as compared with spontaneous recovery from HBV infection. Candidate epitopes were tested for binding to 14 HLA-DR molecules and in IFN-γ ELISPOT and cytotoxicity assays using peripheral blood lymphocytes from 66 HBV-infected patients and 16 uninfected controls. All 10 epitopes bound with high affinity to the most prevalent HLA-DR Ags, were conserved among HBV genomes, and induced IFN-γ responses from HBV-specific CD4⁺ T cells. Several epitopes contained nested MHC class I motifs and stimulated HBV-specific IFN-γ production and cytotoxicity of CD8⁺ T cells. HBV polymerase-specific responses were more frequent during acute, self-limited hepatitis and after recovery (12 of 18; 67%) than during chronic hepatitis (16 of 48 (33%); p = 0.02). Antiviral therapy of chronic patients restored HBV polymerase and core-specific T cell responses during the first year of treatment, but thereafter, responses decreased and, after 3 years, were no more frequent than in untreated patients. Decreased T cell responsiveness during prolonged therapy was associated with increased prevalence of lamivudine-resistant HBV mutants and increased HBV titers. The data provide a rationale for the combination of antiviral and immunostimulatory therapy. These newly described HBV polymerase epitopes could be a valuable component of a therapeutic vaccine for a large and ethnically diverse patient population. The Journal of Immunology, 2004, 173: 5863–5871.

the hepatitis B virus (HBV) is a noncytopathic DNA virus that causes both acute and chronic liver disease. Despite the availability of an effective vaccine for more than two decades, hepatitis B remains one of the 10 most common causes of death worldwide. More than 5% of the world population, i.e., 400 million people, are currently infected with HBV, and >250,000 people die each year from HBV-related liver cirrhosis and hepatocellular carcinoma (1). The development of antiviral and immunostimulatory therapies for HBV-infected patients therefore remains an important priority.

Recovery from acute hepatitis B is the result of a combination of cellular and humoral immune responses. Whereas neutralizing Abs against hepatitis B (HB) virus surface (s) Ag (HBsAg) appear after recovery, cellular immune responses are generally detectable before the synthesis of neutralizing Abs (2) and precede the rise of serum alanine aminotransferase (ALT) levels (3) as well as clearance of HBeAg and HBsAg. They are mediated by cells of the innate immune response (4, 5) and by CD4⁺ and CD8⁺ T cells that clear HBV by cytolitic (6) and noncytolytic, cytokine-mediated (4, 7) mechanisms. After recovery, HBV-specific T cells persist in the blood for decades (8). In contrast, in chronic HBV infection, HBV-specific cellular immune responses are typically weak, narrowly focused, and rarely detectable in the peripheral blood (2, 9–11). However, they are not completely absent, because transient increases in the cellular immune response have been shown to precede increases of ALT activity and can be followed by the development of neutralizing Abs and spontaneous recovery (12). Therefore, it has been suggested that therapeutic induction of HBV-specific cellular immune responses may lead to recovery from chronic hepatitis B, and a lipopeptide-based vaccine with an HLA-A2-restricted HBV CD8⁺ T cell epitope and a tetanus toxoid CD4⁺ T cell epitope has been evaluated as experimental vaccine (13, 14). Although CD8⁺ T cell responses against the HBCore peptide could be induced in healthy, uninfected controls (15) as well as in patients with chronic hepatitis B (16), this was not sufficient to mediate viral clearance.

Because HBV-specific CD4⁺ T cells contribute to induction (17) and maintenance of Ag-specific CD8⁺ T cells, license dendritic cells to activate CD8⁺ effector T cells (17, 18), and provide help for activation and differentiation of B cells, the induction of HBV-specific CD4⁺ T cells is regarded as an important component of any immunomodulatory therapy. Whereas CD4⁺ T cell epitopes have been identified in the HBCore, HBs, and HBe Ag, the largest protein, HBV polymerase, has not been studied. This is a significant omission because HBV polymerase is a highly immunogenic CD8⁺ T cell target in acute self-limited hepatitis, even though it is produced in significantly smaller quantities than the other HBV Ags (2). Because polymerase is essential for the earliest steps in the
HBV life cycle, recognition of this Ag may limit early HBV spread, and its high degree of conservation may prevent viral escape via mutations in T cell epitopes. Finally, only polymerase-encoding plasmids and not envelope-encoding plasmids were able to break tolerance and to induce specific CD8+ T cells upon immunization of HBV transgenic mice (19). The characterization of CD4+ T cell responses against HBV polymerase, which is performed in the current study, does therefore fill an important gap in our understanding of the HBV-specific immune response and is relevant for the development of multiepitope, immunostimulatory vaccines.

A second issue that is addressed by the current study is the effect of antiviral therapy on T cell responsiveness. Whereas some studies reported a restoration of HBV-specific T cell responses when persistently infected patients were treated with nucleoside analogs that inhibit HBV replication (20, 21), these results have not been confirmed in other studies (22), and most treated patients do not maintain viral control (23, 24). Thus, the hypothesis that high levels of HBV may suppress HBV-specific T cell responses is still controversial. Studying 66 patients with past or present HBV infection, we show that HBV polymerase-specific responses correlated with clinical and serological recovery from hepatitis B and infection, we show that HBV polymerase-specific responses correlated with clinical and serological recovery from hepatitis B and infection, we show that HBV polymerase-specific responses correlated with clinical and serological recovery from hepatitis B and infection, we show that HBV polymerase-specific responses correlated with clinical and serological recovery from hepatitis B and infection, we show that HBV polymerase-specific responses correlated with clinical and serological recovery from hepatitis B and infection.

Materials and Methods

Patient population

Sixty-six adult patients with past or present HBV infection were grouped as follows: group 1, 1 patient with acute, self-limited hepatitis B, who was positive for HBsAg and anti-HBc in serum served as controls. Group 2, 17 recovered patients who were negative for HBsAg, but reactive with various unlabeled HBV peptides and 1–10 nM 125I-radio-labeled probe peptides for 48 h. Assays were performed at pH 7.0 with the exception of that for DRB1*0301, which was performed at pH 4.5.

<table>
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<tr>
<th>Peptide Number</th>
<th>HBV Protein</th>
<th>Amino Acid Position</th>
<th>Amino Acid Sequencea</th>
<th>Conservationb (%)</th>
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<td>50–70</td>
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<td>767–782</td>
<td>AANJILGRTSFVYPV</td>
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a Amino acid sequences of nested MHC class I binding motifs are underlined.

b Sequence conservation among 20 full-length HBV genomes in the GenBank database, including adr, adw, ayr, and ayw isolates.

Laboratory and virologic testing

Serum HBsAg, HBeAg, and anti-HBe were detected with commercial immunoassays (Abbott Laboratories, Abbott Park, IL). Molecular typing of HLA DR alleles was performed on genomic DNA using standard site-specific oligonucleotide PCR. Serum HBV DNA was quantitated by branched DNA signal amplification assay (Chiron Corporation, Emeryville, CA) with a lower limit of detection of 0.7 × 10<sup>6</sup> genome equivalents (10<sup>6</sup> genome equivalents = 1 nEq/ml) per milliliter (26). Samples with HBV DNA of <1 nEq/ml were tested by quantitative PCR (National Genetics Institute, Los Angeles, CA) with a lower limit of detection of 100 copies/ml and by qualitative nested PCR as described (8). RFLP assay was used to detect virological lamivudine resistance defined by either YIDD mutants (methionine-to-isoleucine substitution at codon 552 (M552I)) or YYVDD mutants (methionine-to-valine substitution at codon 52 (M552V)) in conjunction with a leucine-to-methionine substitution at codon 52 (M528M) of the HBV polymerase gene (24, 27, 28) as previously described (25). The lower limit of detection of this method was ~500 copies viral DNA/ml serum.

Synthetic peptides

MHC class II-restricted peptides were synthesized at Epimmune on an Applied Biosystems (Foster City, CA) 430A peptide synthesizer using 9-fluorenylmethoxy carbonyl chemistry and purified to >95% purity by reverse-phase HPLC. MHC class I-restricted peptides were synthesized at Research Genetics (Huntsville, AL) at >80% purity.

Purification of HLA class II molecules

HLA class II molecules were purified from the EBV-transformed homozygous cell lines LG2 (alleles DRB1*0101 (Ag DR1)); MAT (DRB1*0301 (DR3)); PREISS (DRB1*0401 (DR4w4)); KT3 (DRB1*0405 (DRw15)); SWIG (DRB1*0101 (DRw11)); Herluf (DRB1*0101 (DRw12)); HO301 (DRB1*1302 (DRw19)); FITOUT (DRB1*0701 (DR7)); OLL (DRB1*0802 (DRw22)); HID (DRB1*0901 (DR9)); GM3107 (DRB5*0101 (DRw22a)); TR81.9 (DRB3*0101 (DRw2a); L257.6 (DRB4*0101 (DRw53)); and from the transfected fibroblast line L466.1 (DRB1*1501 (DRw2b)) (29). Large quantities of cells were grown in spinner cultures in RPMI 1640 with 2 mM l-glutamine (Invitrogen Life Technologies, Carlsbad, CA), 100 μg/ml streptomycin, 100 U/ml penicillin (Irvinie Scientific, Santa Ana, CA), 50 μM 2-ME, and 10% heat-inactivated FCS (Calbiochem, La Jolla, CA). Lysates were collected from these cultures with buffers containing 15,000 × g for 30 min. Class II molecules were purified by affinity chromatography using the mAb LB3.1 coupled to Sepharose CL-4B beads as previously described (30, 31).

HLA-DR peptide-binding assays

Fourteen different purified human HLA-DR molecules (5–500 nM) were incubated with various unlabeled HBV peptides and 1–10 nM 125I-radio-labeled probe peptides for 48 h. Assays were performed at pH 7.0 with the exception of that for DRB1*0301, which was performed at pH 4.5.
Table II. HLA-DR binding capacity (IC50 nM)

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<th>DRB1*0802</th>
<th>DRB1*0901</th>
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**Notes:**
- **IC50**: Half-maximal inhibitory concentration.
- **DR**: Dendritic cell.
- **n**: Number of DR alleles bound.
- **IC50**: Concentration in nM.
- **%**: Percentage of binding.

HLA-DR peptide complexes were separated from free peptide by gel filtration on TSK200 columns (TosoHaas, Montgomeryville, PA), and the fraction of bound peptide was calculated as previously described (31). In preliminary experiments, the titers of the HLA-DR preparation were determined in the presence of fixed amounts of radiolabeled peptides to determine the concentration of HLA-DR molecules necessary to bind 10–20% of the total radioactivity. Peptide inhibitors were typically tested at concentrations ranging from 1.2 ng/ml to 120 μg/ml. All subsequent inhibition and direct binding assays were then performed using these HLA-DR concentrations.

The radiolabeled probes were HA Y307-319 for DRB1*0101; TT830-843 for DRB5*0101, DRB1*1101, DRB1*0701, DRB1*0802, and DRB1*0901; MBP Y85-100 for DRB1*1501; MT 65 KD Y3-13 with Y7 replaced with F for DRB1*0301; a nonnatural peptide with the sequence YARFQRQTLKLQK for DRB1*0401 and DRB1*0405; a nonnatural peptide with the sequence YARFQRQTLKLK for DRB4*0101 (29); a naturally processed peptide of unknown origin eluted from a DRB1*1201* CIR cell line with the sequence EALIHQLKNYPVSLS (32), an analog of TT830-843 with the sequence YIKANAKFYGTE for DRB1*1302 (33); and integrin β1, Y24-37 with the sequence YAWASDEALPGLPS for DRB3*0101.

**ELISPOT assay**

IFN-γ ELISPOT assays were performed as previously described with duplicate cultures of 3 × 10⁵ freshly isolated PBMC (34), 10 μg/ml human HBV core protein (ViroGen, Watertown, MA), 50 μg/ml tetanus toxoid (University of Massachusetts Medical School, Jamaica Plain, MA), or 1 μg/ml PHA (Murex Biotech Limited, Dartford, U.K.) in RPMI 1640 containing 5% AB serum and 2 mM l-glutamine. Stimulation with PHA always resulted in a vigorous response with spots too numerous to count. In selected experiments, 10⁶ CD4⁺ or CD8⁺ T cells, purified with anti-human CD8 Ab-conjugated magnetic microbeads (Dynal, Oslo, Norway), were cultured with 2 × 10⁵ autologous irradiated (3000 rad) PBMCs as APCs in the presence or absence of Ags. Control experiments confirmed that the irradiated PBMC fraction did not produce any detectable IFN-γ spots. The number of specific spots (spots in the presence of Ag minus spots in the absence of Ag) was determined with a KS ELISPOT reader (Zeiss, Thornwood, NY), and represented the number of spots in the absence of Ag from the number of spots in the presence of Ag.

PBMC of 16 healthy, anti-HCV negative control persons who were negative for serum HBV DNA, HBsAg, and anti-HBe were tested with the same peptides with the following results (mean number of spot-forming cells + SD per 3 × 10⁵ PBMC): peptide 1, 1.31 ± 2.4; NS3, peptide 2, 0.56 ± 1.2; peptide 3, 2.25 ± 4.2; peptide 4, 1.1 ± 1.2; peptide 5, 1.25 ± 2.7; peptide 6, 0.31 ± 1; peptide 7, 1.94 ± 2.8; peptide 8, 0.44 ± 1.1; peptide 9, 2.06 ± 2.8; peptide 10, 2.56 ± 4.1; peptide 11, 0.31 ± 0.8; and peptide 12, 0.81 ± 1.4. A response was scored as positive if it was 1) greater than the mean response plus 2 SD in healthy, anti-HCV negative control subjects and 2) greater than 10 specific spots (spots in the presence of Ag minus spots in the absence of Ag) (35).

**Cytotoxicity analysis using nested CD8⁺ T cell epitopes**

HBV-specific CD8⁺ T cells were expanded from PBMC by stimulation with the nested peptides that contained HLA-A2 and HLA-A3 binding motifs (Table I). Cytotoxicity was assessed after 3 wk of culture using 25-μCi-labeled, peptide-pulsed HMBCIR cells transfected with HLA-A2.1 or autologous PHA-stimulated T cell blasts as previously described (34). Spontaneous release was <15% of maximum release in all experiments.

**Statistical analysis**

Fisher’s exact test (two-tailed) was used to compare the frequency of immune responses in different patient groups. Student’s t-test (two-tailed) was used to compare the level of ALT and HBV DNA in different patient groups. A value of p < 0.05 was considered significant.

**Results**

**Selection of highly cross-reactive HLA-DR binding peptides**

Ten HBV polymerase peptides that contained either the HLA-DR supermotif (36) (peptides 5, 7, 9–11, and 13) or the DR3 motif (37) (peptides 4, 6, 8, and 12) were tested for binding to a panel of HLA DR B1, B3, B4, and B5 alleles (38). These HLA-DR molecules were selected as representative of the most common HLA variants expressed in the majority of the world’s ethnic population.
The sequences of most selected HBV polymerase peptides were conserved in >80% of the 20 most common HBV strains including adr, adw, ayr, and ayw isolates (Table I). In addition, several peptides that were conserved in only ~50% of isolates, but highly conserved in their DR binding region, were included in the analysis. For comparison, we also studied one HBV nucleocapsid (39) and two HBV envelope peptides (40, 41) (Table I) and the HBc core protein (see Fig. 4).

As shown in Table II, all HBV peptides bound with an IC50 of ≤1000 nM to at least one HLA-DR molecule. The IC50 value represents the amount of peptide required for 50% inhibition of binding of a fluorescein-labeled reference peptide. An IC50 value of 1000 nM has previously been shown to represent the affinity threshold associated with immunogenicity (38, 42). Moreover, 8 of the 10 polymerase peptides bound at least four of these frequent DR molecules. This degree of cross-reactivity is not dissimilar from the previously identified control peptides (39–41), and suggested that these peptides should be recognized by subjects with diverse HLA haplotypes. Based on the worldwide prevalence of the HLA-DR Ags, this panel of peptides should cover 98.2% of the average population.

Immunogenicity of HBV polymerase peptides as assessed by IFN-γ ELISPOT analysis
To determine whether these highly cross-reactive and conserved HLA-DR binding peptides were recognized by T cells of patients with past or present HBV infection, IFN-γ ELISPOT responses were evaluated with PBMC from 1 acutely HBV-infected patient, 17 recovered patients, and 48 persistently HBV-infected patients (Table III). Sixteen blood donors were tested as controls. The cut-off of positivity was set at ≥10 IFN-γ spots/300,000 PBMC (see Materials and Methods). All 10 HBV polymerase peptides were recognized by PBMC of at least one patient, and 29 of 66 patients (44%) responded to at least one of the analyzed HBV polymerase peptides (Fig. I). Peptides 4 and 8 were the only peptides that were exclusively recognized by the patient with strongest immune response and acute hepatitis B, and one of these peptides (peptide 4) had a very low HLA-DR binding affinity (Table II). By contrast, peptides 7 and 11, associated with significant binding to 77 and 92% of the HLA-DR molecules, respectively, were recognized by 8 and 13 patients. These data demonstrate that conserved HBV polymerase peptides selected on the basis of a high binding affinity to several HLA DR molecules are antigenic in the context of multiple DR alleles, and thus allow coverage of a broad and ethnically diverse patient population.

In the total patient population, 61 responses were observed against HBV polymerase peptides. The patients’ HLA haplotypes were known for 56 of these 61 cases. When the observed responses were evaluated in the context of the patients’ HLA haplotypes, 55% (31 of 56) of all responses occurred in the presence of one of the DR alleles with high peptide-binding affinity (IC50 < 1000 nM; Table II). In an additional 20% (11 of 56) of all responses, the patients expressed DR molecules at least weakly binding (1000 nM < IC50 < 20 μM) the peptide. In the remaining cases, patients did not express any DR alleles known to bind the peptide (IC50 < 20 μM; Table II), suggesting that some of the responses might be restricted by other alleles not analyzed in the current study.

The magnitude of the HBV-specific T cell responses was assessed by the frequency of HBV nucleocapsid, envelope and polymerase peptide-specific, IFN-γ-producing T cells in the PBMC population (Fig. 2). The broadest T cell response was noted during acute, self-limited hepatitis B with responses to all 13 HBV epitopes. In contrast, none of the other patients responded to more than five epitopes, and the overall response of patients with chronic hepatitis B was weak. As previously reported for other HBV epitopes and proteins (35, 43, 44), the frequency of HBV epitope-specific cells was low, i.e., between ~10 and 50 peptide-specific cells per 300,000 PBMC. Tetanus toxoid-specific responses were tested as a positive control, and the frequency of responses did not differ significantly among patient subgroups (Fig. 2C).

T cell subsets responding to HBV polymerase peptides
To determine the T cell subset that responded to the HBV polymerase peptides, PBMC were separated into CD4+ and CD8+ subpopulations using Ab-coated magnetic beads. Each cell subpopulation was then separately tested in the presence of irradiated, autologous APCs and the respective peptides. Fig. 3A demonstrates in a representative experiment that most IFN-γ spots in the ELISPOT assay segregated with the CD4+ T cell subfraction. However, in the case of peptide 9, ~25% of the total number of IFN-γ spots could still be attributed to CD8+ T cells (Fig. 3A). A closer analysis of the peptide sequences revealed that peptide 9 as well as four other polymerase peptides contained nested HLA class I-binding motifs (Table I). In fact, nested sequences within peptides 5, 6, and 11 have previously been described as CD8+ T cell epitopes (45). Thus, in natural HBV infection, these shorter peptides may be endogenously processed from polypeptide precursors, loaded onto HLA class I molecules in HBV-infected cells, transported to the cell surface, and stimulate responses of CD8+ T cells.

To test this hypothesis, we chose the nested sequences HLY-SHPiII in peptide 9 and QAFTFSPTYK in peptide 11, which contained the HLA-A2 and HLA-A3 binding motifs, respectively, and tested them for CTL recognition using a microwell peptide stimulation technique to expand low-frequency HBV-specific CTL.

Table III. Baseline characteristics of the patients studied

<table>
<thead>
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<th>Group</th>
<th>Clinical Diagnosis</th>
<th>No. of Patients</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>ALT (IU/L)</th>
<th>HBsAg+ (%)</th>
<th>HBV DNA (copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acute HBV patient</td>
<td>1</td>
<td>1/0</td>
<td>27</td>
<td>641</td>
<td>1 (100)</td>
<td>1.5 × 10^4</td>
</tr>
<tr>
<td>2</td>
<td>Recovered patients</td>
<td>17</td>
<td>15/2</td>
<td>52 ± 9</td>
<td>55 ± 12</td>
<td>0 (0)</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Chronic hepatitis B, no therapy</td>
<td>3a</td>
<td>HBeAg positive</td>
<td>7</td>
<td>6/1</td>
<td>45 ± 21</td>
<td>97 ± 58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3b</td>
<td>HBeAg negative</td>
<td>7</td>
<td>4/3</td>
<td>49 ± 9</td>
</tr>
<tr>
<td>4</td>
<td>Chronic hepatitis B, on therapy</td>
<td>4a</td>
<td>HBeAg positive</td>
<td>20</td>
<td>18/2</td>
<td>46 ± 3</td>
<td>84 ± 82</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4b</td>
<td>HBeAg negative</td>
<td>14</td>
<td>12/2</td>
<td>49 ± 13</td>
</tr>
</tbody>
</table>

* M, Male; F, Female.
* Negative by branched DNA analysis and nested PCR.
* Lamivudine therapy (100 mg orally per day).
precursors from PBMC. Overall, the HLA-A2 restricted epitope in peptide 9 and the HLA-A3-restricted epitope in peptide 11 expanded HBV-specific CD8+ T cells of several patients with the corresponding HLA class I haplotype (Fig. 3B). These results demonstrate that several of the newly identified CD4+ T cell epitopes contained shorter sequences that were recognized by CD8+ T cells when presented as the minimal optimal epitope in the context of the matching HLA class I molecule.

Cross-sectional analysis of HBV polymerase-specific T cell responses in patient groups with different outcome of HBV infection

Because HBV polymerase is an essential enzyme for HBV replication, expressed early in the HBV life cycle and also found inside the secreted HBV particle, we reasoned that the frequency of HBV-specific T cell responses might correlate with different stages of HBV infection. As previously shown for CD8+ T cell responses against polymerase (2, 3), there was a significant difference in the frequency of CD4+ T cell responses between the different patient subgroups. Whereas the patient with acute, self-limited hepatitis B recognized all epitopes, and 11 of 17 (65%) recovered patients (group 2) tested positive for at least one HBV polymerase peptide, only 16 of 48 (33%) of patients with chronic hepatitis B (groups 3 and 4) tested positive (p = 0.043; Fig. 1). When immune responses against any peptide, i.e., including the nucleocapsid and envelope peptides, were considered, this difference between acutely infected and recovered patients (16 of 18 (89%) responding) and chronically infected patients (19 of 48 (40%) responding) was even more significant (p < 0.0001; Fig. 1).

Among patients with chronic hepatitis B, further analysis was performed to assess the role of lamivudine therapy on cellular immune responses to HBV polymerase peptides. Cellular immune responses to HBV polymerase were detected in 4 of 14 untreated (29%) and in 12 of 34 (35%) treated patients. Within the lamivudine-treated group, the duration of treatment at the time of testing appeared to correlate with reactivity. Four of 5 (80%) patients treated for 1 year responded to the polymerase epitopes as compared with 3 of 7 (43%) patients treated for 1–3 years, 5 of 22 (24%) patients treated for 3–5 years, and 4 of 14 (29%) patients who were not treated (Fig. 4A). These differences in the polymerase-specific responses between the subgroups were statistically significant (p = 0.029 for 1-year treatment vs 3-year treatment; p = 0.041 for 1-year treatment vs 1-3-year treatment) (Fig. 4A) and correlated with differences in viral levels among patient subgroups. For example, the highest frequency of HBV polymerase-specific T cell responses was observed in patients who had received lamivudine therapy for 1 year and displayed the lowest HBV DNA levels. In contrast, HBV polymerase-specific T cell responses were lower in patients who had been treated for 1–3 and for 3–5 years, respectively, and these patient subgroups were characterized by 100- to 1000-fold higher HBV DNA levels due to development of lamivudine-resistant HBV mutants (Fig. 4A).

### Table 1

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Pt. No.</th>
<th>Nucleocapsid and Envelope Peptides</th>
<th>Polymerase Peptides</th>
<th>Response Frequency per Peptide</th>
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</thead>
<tbody>
<tr>
<td>1 Acute, self-limited</td>
<td>1</td>
<td>1</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>2 Recovered</td>
<td>2</td>
<td>5</td>
<td>8%</td>
<td>92%</td>
</tr>
<tr>
<td>3a Chronic, untreated</td>
<td>3</td>
<td>13</td>
<td>6%</td>
<td>94%</td>
</tr>
<tr>
<td>3b Chronic, untreated</td>
<td>4</td>
<td>15</td>
<td>8%</td>
<td>92%</td>
</tr>
<tr>
<td>4a Chronic, treated</td>
<td>5</td>
<td>11</td>
<td>8%</td>
<td>92%</td>
</tr>
<tr>
<td>4b Chronic, treated</td>
<td>6</td>
<td>7</td>
<td>8%</td>
<td>92%</td>
</tr>
</tbody>
</table>

*FIGURE 1. HBV nucleocapsid, envelope, and polymerase peptide-specific T cell responses. Only patients who recognized at least one of the 13 tested peptides in the IFN-γ ELISPOT assay are shown. A positive IFN-γ response was defined as >10 spot-forming cells per 300,000 PBMC, which is greater than the mean plus 2 SD of the baseline response detected in 16 healthy uninfected controls. Shaded boxes indicate the presence of a significant IFN-γ T cell response to HBV nucleocapsid and envelope peptides; black boxes indicate the presence of a significant IFN-γ T cell response to HBV polymerase peptides. Peptide sequences are described in Table I, and patient groups in Table III.*
Overall, the frequency of HBV polymerase-specific responses was higher in patients who had successfully cleared HBeAg (Fig. 4B) than in patients who remained HBeAg positive under lamivudine therapy (B). When HBeAg− and HBeAg+ patients were analyzed separately, HBV polymerase-specific T cell response decreased in both subgroups under prolonged lamivudine therapy. Consistent with the findings for all treated patients (group 4), this reduced T cell responsiveness correlated with increased HBV DNA levels and emergence of lamivudine resistance mutants, but did not reach statistical significance due to the small number of patients in each subgroup (Fig. 4B). Finally, a similar trend toward decreased HBV-specific immune responsiveness under prolonged lamivudine therapy was observed for HBeAg− patients with acute, self-limited hepatitis B (2, 3). Third, the newly

**Discussion**

The current study uses a combined immunochemical and cellular immunology approach to identify and characterize 10 highly conserved and immunogenic CD4+ T cell epitopes in the HBV polymerase protein. The newly identified CD4+ T cell epitopes within HBV polymerase are relevant for several reasons. First, all epitopes displayed significant binding affinity to multiple HLA-DR molecules. Therefore, the data suggest that the majority of the world’s population, independent of ethnicity, should be able to respond to these epitopes. Second, CD4+ T cell epitopes within HBV polymerase offer significant advantages for the development of epitope-based diagnostics and vaccines, because of the high degree of sequence conservation of HBV polymerase and its essential role in the viral life cycle, which reduces the possibility of viral escape by mutations in T cell epitopes. In addition, HBV polymerase represents the largest HBV protein and has already been shown to be highly immunogenic at the CD8+ T cell level in patients with acute, self-limited hepatitis B (2, 3). Third, the newly

**FIGURE 2.** Vigor of HBV nucleocapsid, envelope, and polymerase peptide-specific T cell responses. A and B, Direct ex vivo IFN-γ ELISPOT analysis of peripheral blood T cell responses to HBV nucleocapsid and envelope peptides (A) and HBV polymerase peptides (B). Only significant IFN-γ responses greater than the mean plus 2 SD (indicated by horizontal line) of the baseline response detected in 16 healthy uninfected controls and >10 specific spots/300,000 PBMC are shown. The asterisk indicates 102 spots. Peptide sequences are described in Table I, patient groups (II, group 1; I, group 2; II, group 3; III, group 4) are defined in Table III. C, Tetanus-specific responses. Responses of >100 spots were too numerous to count with the automated ELISPOT reader.

**FIGURE 3.** Identification of nested MHC class I-restricted epitopes. A, PBMC were sorted into CD4+ and CD8+ T cell subpopulations using Ab-coated magnetic beads and stimulated separately with peptide-pulsed autologous APCs. The number of peptide-specific, IFN-γ-producing cells as determined by ELISPOT analysis is indicated and greater for the CD4+ T cell subpopulation than for the CD8+ T cell subpopulation. A representative experiment is shown. B, Cytotoxic CD8+ T cells specific for MHC class I-restricted peptides that are nested within the HBV-polymerase CD4+ T cell epitopes. T cell lines were generated from PBMC of the HLA-A2-positive patients 12, 29, and 39, and the HLA-A3-positive patient 17, respectively, by stimulation with the nested 9- and 10-mer peptides located within peptide 9 (●) or 11 (■) (see Table I). Expanded T cell lines were then tested for specific cytotoxicity against the nested peptides in a standard 51Cr release assay at the indicated E:T ratios.
identified HBV polymerase epitopes were able to induce IFN-γ production by HBV-specific T cells, an effector function associated with in vivo suppression of HBV replication (7, 46). Fourth, the observation that several of these CD4+ T cell epitopes contain nested MHC class I-restricted epitopes that stimulate IFN-γ responses and cytotoxicity by CD8+ T cells is an additional attractive feature for a multi-epitope vaccine. The well-characterized, immunodominant HBc18-27 CD8+ T cell epitope, for example, does also overlap with an MHC class II-restricted CD4+ T cell epitope (47), and it has been described that CD4+ T cells are indispensable for the maintenance of functional CD8+ T cells that control chronic viral infections (48). This CD4+ T cell help may consist of direct help for CD8+ T cells via production of cytokines (49) or of indirect help for professional APCs via CD40/CD40L-mediated activation (50). Moreover, recent studies have demonstrated the need to include potent CD4+ T cell epitopes to restore an altered Th response and to overcome CD8+ T cell tolerance in chronic HBV infection (14). Because covalent linkage of CD4+ and CD8+ T cell epitopes on the same peptide vaccine construct is important for the induction of Ag-specific responses (51), the natural occurrence of nested CD8+ and CD4+ T cell epitopes within the polymerase Ag might provide an elegant and promising approach to fulfill these requirements.

Finally, the presence of HBV polymerase-specific, CD4+ T cell responses correlated with different outcomes of HBV infection. As demonstrated for CD8+ T cell responses (2), circulating HBV polymerase-specific CD4+ T cell responses were more frequent in acute self-limited hepatitis B and after recovery than in chronic hepatitis B. Moreover, even in the persistently infected, HBsAg+ patient subgroup, HBV polymerase-specific T cell responses were associated with a partial response, i.e., with loss of HBeAg (Fig. 4B) and reduction of HBV DNA levels (A). Although the recognition of endogenously processed HBV polymerase could not be tested due to a lack of recombinant and purified polymerase protein, these observations and the absence of those responses in healthy, uninfected blood donors indicated that all epitopes were processed in vivo and that HBV polymerase-specific CD4+ T cells were primed in vivo.

In the second part of this study, the newly identified CD4+ T cell epitopes were then used to analyze the HBV-specific immune response during antiviral therapy. Recent studies have suggested that suppression of HBV replication by lamivudine rapidly restores HBV-specific cellular immune responsiveness to the same level as in recovered persons (20). In that study, both CD4+ (20) and CD8+ (21) T cells specific for HBV remained detectable in the blood for up to 5 mo during lamivudine therapy. However, the increased T cell reactivity was not associated with an increase in loss of HBeAg or HBsAg (20), the serological hallmark of recovery from hepatitis B. Also, a second study of patients receiving IFN-α alone and in combination with lamivudine reported no restoration or de novo induction of HBV core-specific T cell proliferation (22). Because HBV polymerase is expressed early in the HBV life cycle and also found inside the secreted HBV particle, we reasoned that the frequency of HBV polymerase-specific T cells might correlate more closely with viral levels and the outcome of lamivudine therapy.

Using the newly identified HBV polymerase peptides, the current study indicated that antiviral therapy of persistently infected patients appeared to increase the frequency of HBV-specific CD4+ T cell responses during the first year of treatment. This result indicates that HBV-specific T cells are not completely depleted or anergized during chronic hepatitis B because their frequency in the
peripheral blood can significantly increase upon therapeutic reduction of HBV levels. The reconstitution of HBV-specific T cells in the periphery may result from generation of new precursors in the regional lymph nodes on the one hand (52) and from decreased responsiveness of effector T cells to the liver.

With longer periods of antiviral therapy, however, the frequency of HBV-specific CD4+ T cell responses decreased and, after 3 years of therapy, responses were no more frequent than in untreated patients. Decreased responsiveness of HBV-specific T cells correlated with the emergence of lamivudine-resistant HBV mutants and a concomitant increase of HBV DNA levels. Whether the lack of HBV-specific responses predated the development of viral resistance and may have contributed to its development, is an interesting question, that could not be answered due to the cross-sectional nature of this study and warrants future, prospective studies. Overall, the results suggest that therapeutic vaccination designed to boost HBV-specific cellular immunity might be most efficient during the first year of antiviral therapy. The degenerate HLA-DR binding and immunogenicity of the HBV polymerase epitopes as well as the inclusion of nested CD8+ T cell epitopes make these epitopes valuable components of a vaccine that is designed to cover a large and ethnically diverse patient population.

Acknowledgments

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References


