Overcoming T Cell Tolerance to the Hepatitis B Virus Surface Antigen in Hepatitis B Virus-Transgenic Mice

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Overcoming T Cell Tolerance to the Hepatitis B Virus Surface Antigen in Hepatitis B Virus-Transgenic Mice

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The sequence of the hepatitis B virus (HBV) major envelope (Env) protein (ayw subtype) was scanned for the presence of H-2D\textsuperscript{b} motifs. Following binding and immunogenicity testing, two new H-2D\textsuperscript{b}-restricted epitopes (Env.362 and Env.364) were identified. These epitopes induced CTLs capable of recognizing naturally processed HBV-Env, but were apparently generated with lower efficiency than the previously defined dominant Env.28 epitope. Next, HBV-transgenic mice that express all of the HBV proteins and produce fully infectious particles were immunized with a mixture of lipopeptides encompassing the Env.28, Env.362, and Env.364 epitopes. Significant CTL responses were obtained, but they had no effect on viral replication in the liver, nor did they induce an inflammatory liver disease. However, in adoptive transfer experiments, CTL lines generated from the HBV-transgenic mice following immunization were able to inhibit viral replication in vivo without causing hepatitis. This is in contrast to CTL lines derived from nontransgenic mice that displayed both antiviral and cytopathic effects, presumably because they displayed higher avidity for the viral epitopes than the transgenic CTLs. These results suggest that T cell tolerance to HBV can be broken with appropriate immunization but the magnitude and characteristics of the resultant T cell response are significantly different from the response in HBV-naive individuals since their antiviral potential is stronger than their cytotoxic potential. This has obvious implications for immunotherapy of chronic HBV infection. The Journal of Immunology, 2001, 166: 1389–1397.

The hepatitis B virus (HBV) is a noncytopathic DNA virus that chronically infects ~350 million people worldwide (1, 2). Adult onset infection usually results in self-limited acute hepatitis followed by viral clearance, although up to 5% of infected adults become chronically infected (2). In contrast, neonatal infection is seldom cleared and a majority of infected children develop persistent infection (2). Patients chronically infected with HBV are predisposed to developing cirrhosis of the liver and hepatocellular carcinoma (2).

CD\textsuperscript{8} T lymphocytes are an important component of host defense mechanisms responsible for HBV clearance in patients with acute hepatitis (3, 4). In particular, multispecific responses appear to be associated with spontaneous resolution of acute infection. In this respect, elicitation of responses directed against multiple epitopes may be of therapeutic interest (2). It has been proposed that elimination of virus is due to CTL-mediated lysis of infected hepatocytes and/or antiviral effects of CTL-derived cytokines, such as IFN-\textgamma and TNF-\alpha (5, 6). Recent data demonstrate that, in acutely infected chimpanzees, HBV DNA is eliminated from the liver before the peak of T cell infiltration and the associated liver disease (7). These effects coincide with the appearance of IFN-\textgamma in the liver, suggesting that IFN-\textgamma-producing non-T cells, presumably NK and NKT cells, as well as T cells, play a key role in the control of HBV infection. Thus, noncytopathic mechanisms that eliminate replicative HBV DNA intermediates from the cytoplasm and covalently closed circular DNA from the nucleus (7) appear to be crucial for clearance of HBV from the hepatocyte. Therapeutic induction of this kind of noncytopathic, antiviral activity in the liver of chronically infected patients would, therefore, lead to viral clearance, thereby preventing cirrhosis and hepatocellular carcinoma.

Unfortunately, chronic HBV infection, like many other chronic viral diseases and cancers (2), is associated with T cell hyporesponsiveness or tolerance. The exact molecular mechanisms associated with this phenomenon are not entirely clear but negative selection, peripheral anergy, and imbalances in lymphokine production all appear to contribute to maintaining the hyporesponsive state of the host chronically exposed to viral or cancer Ags (2).

Transgenic mice of the 1.3.32 lineage contain the complete HBV genome, express all HBV gene products, and replicate HBV in their hepatocytes similar to chronically infected patients, but they do not develop chronic hepatitis because they are immunologically tolerant to the viral Ags (5, 8). The availability of these mice provides a model system to evaluate immunotherapeutic strategies to break tolerance and terminate persistent HBV infection.

Thus far, several approaches to overcome CTL tolerance in HBV-transgenic mice have been investigated. In previous studies, five different lineages of HBV-transgenic mice, including lineage 1.3.32, were immunized with either plasmid DNA encoding whole HBV Ags or activated dendritic cells. DNA immunization induced Ab responses in some of the lineages, but no CTL responses (8). Dendritic cell immunization generated CTLs but they had no effect on viral replication and they did not cause hepatitis (8). These results are in contrast with a previous study which had indicated

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\footnotesize{\textsuperscript{3}Abbreviations used in this paper: HBV, hepatitis B virus; Env, envelope; HTL, helper T lymphocytes; sALT, serum alanine aminotransferase; LCMV, lymphocytic choriomeningitis virus.}

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that tolerance could be broken by DNA immunization in a single lineage of transgenic mice that contain a subgenomic fragment of HBV and express the major envelope (Env) protein within hepatocytes (9). In these animals, DNA immunization was associated with a reduction of HBV RNA expression in hepatocytes (9) and this effect was shown to be dependent on IFN-γ. The immunological, virological, and clinical relevance of these results is uncertain; however, the transgene in the particular lineage of mice used in those experiments is susceptible to genomic imprinting and silencing of transgene expression by methylation (10). Therefore, these events may have been triggered by immunization but do not occur during infection.

In the present study, we used the complete HBV genome transgenic mouse model to evaluate the relative immunogenicity of a pool of HBV-specific lipopeptides with optimized helper T lymphocyte (HTL) function for their capacity to break T cell tolerance and to down-regulate HBV gene expression and replication.

Materials and Methods

Peptides and lipopeptides

Peptides were synthesized according to standard F-moc solid-phase synthesis methods (11). In HTL-CTL peptide constructs, the HTL epitope was placed at the amino terminus of the CTL epitope. In the case of the lipopeptide synthesis, each amino acid coupling was followed by a capsing cycle with DMTS and dimethylformamide (v/v) to facilitate subsequent purification. Lipopeptides were prepared by coupling preformed symmetrical anhydride of palmitic acid to the amino terminus of the resin-bound KSS-elongated peptide. The peptides and the protecting groups on the amino acids were cleaved using trifluoroacetic acid, ethanedithiol, water (9.5:2.5:2.5, v/v/v), or trifluoroacetic acid, thioanisole, phenol, ethanedithiol, water (10 ml:0.5 ml:0.75 g:0.25 ml:0.5 ml), depending upon the specific protecting group present on the various amino acids.

Unlipilidated peptides were purified using conventional HPLC methods. Lipopeptides were purified by repetitive washes with 50% acetic acid: water (v/v), followed by two washes with H2O and lyophilization. Lipopeptides were characterized by reverse phase-HPLC and mass spectrometry. Reverse-phase-HPLC was performed using Waters analytical HPLC (polymer reverse phase 4.6 × 150 mm, 5 μM, 300 Å, column; Polymer Laboratories, Shropshire, U.K.) at a column temperature of 80°C and detection at 214 nm. The mobile phase buffers were A (water with 0.1% trifluoroacetic acid, containing 1% Nonidet P-40 (Fluka Biochemika, Buchs, Switzerland), 150 mM NaCl, 5 mM EDTA, and 2 mM PMSF) and B (acetonitrile with 0.1% trifluoroacetic acid), and the elution was effected with a gradient of 20–100% B over 30 min at a flow rate of 1 ml min−1. Purity and identity of all compounds were checked by analytical HPLC and mass spectrometry. Purity was ≥50% for lipopeptides and ≥90% for unlipilidated peptides.

Cell lines, Abs, and MHC purification

The mouse lymphoma EL-4 was used as the source of Kb and Dd class I molecules. EL-4 cells were maintained as described previously (12, 13). The mAbs used for purification were Y3 (anti-Kb) and 28-14-8S (anti-Dd) antibodies. Protein purity and effectiveness of depletion steps were monitored by sequential passage over either 34-5-8S, SF1.1.1, or 28-14-8S columns. Cell lysates for MHC purification were treated with a mixture of protease inhibitors. Reverse phase-HPLC was performed using Waters analytical HPLC (150 mm, 5 μM, 300 Å, column) and a mixture of protease inhibitors. At the end of the incubation period, the percentage of MHC-bound radioactivity was determined by size exclusion gel filtration chromatography on a TSK 2000 column (TosoHaas, Montgomeryville, PA).

The concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe (IC50) in competitive inhibition assays was calculated. Peptides were usually tested at one or two high doses, and the IC50 values of peptides yielding positive inhibition were determined in subsequent experiments, in which two to six additional dilutions were tested, as necessary. MHC concentrations yielding ~15% binding of the radiolabeled probe peptide were used for all competition assays. Each competitor peptide was tested in two to four independent experiments. The radiolabeled probes used, and their average IC50 values in the respective assays, are as follows: HIV-111B Env gpG4 > Y analogue (sequence RGPYRAFVTI), 2e1 in Dd for Dd; 1079.03 (sequence KFNPMKYTI), 1.1 nM for Kd, and B35CON2 (sequence PFPKYAAAF), 30 nM for Ld; adenovirus E1A P Y analogue (sequence SGPSTNTVE) 4.4 nM for Dd, and VSV NP52-59 (sequence RGYVFGQL), 3.1 nM for Kd.

Mice

Eight- to 12-wk-old mice were used in all experiments. (C57BL/6 × B10D2F2)F1 (B6D2F1), and (C57BL/6 × BALB/c)F1 (CB6F1) were purchased from The Jackson Laboratory (Bar Harbor, ME). HBV-transgenic mice from lineage 1.3.32 (official designation Tg(HBV 1.3 genome))125 (inbred C57BL/6) were bred at The Scripps Research Institute. Transgenic mice from lineage 1.3.32 have been previously described (17). The hepatocytes from these animals express all of the HBV gene products and replicate HBV at high levels in the liver without any evidence of cytopathology. Lineage 1.3.32 C57BL/6 (H-2b) were backcrossed to BALB/c (H-2d) mice to produce H-2bxd F1 (CB6F1) hybrids for this study.

Peptide formulations and immunizations

Peptides and lipopeptides were resuspended at 20 mg ml−1 and 10 mg ml−1, respectively, in DMSO/1% trifluoroacetic acid and stored as stock solutions at −20°C. Two different formulations were used for immunizations. For experiments involving IFA, each peptide (or peptide mixture) stock solution was diluted after vortexing for 30 s with appropriate amounts of DMSO and PBS at room temperature to obtain the desired peptide concentrations in a final buffer composition of 90% PBS, 10% DMSO/0.1% trifluoroacetic acid. These samples were emulsified with an equal volume of IFA (Difco, Detroit, MI) for 20 min in a 5100 Spemrixiner/mill (Spex Industries, Metuchen, NJ). In experiments involving lipopeptides, stock solutions were heated for 10 min at 45°C (to ensure solubilization of the lipopeptides in DMSO) and then vortexed for 30 s before dilution with PBS at room temperature. Eight- to 12-wk-old mice were immunized s.c. in the base of the tail with the formulations described above.

Assay of CTL activity

Eleven to 14 days after immunization, splenocytes were harvested from individual mice and 3 × 107 cells were stimulated with specific HBV peptides at 10.0 μg ml−1 in the presence of 105 irradiated (4500 rad) syngeneic LPS blasts. To obtain LPS blast cells, syngeneic splenocytes were resuspended at a concentration of 1–1.5 × 107 cells ml−1 in complete media in the presence of 25 μg ml−1 of LPS (Sigma, St. Louis, MO) and 7 μg ml−1 of dextran sulfate (Pharmacia Biotech, Uppsala, Sweden) and kept in culture for 72 h at 37°C. After 5–6 days, splenocytes from each flask were collected and assayed for cytolityc activity using a standard 4-h 51 Cr release assay.

The following cell lines were used as target cells in the cytotoxicity assays: EL4 (a murine lymphoma), P815 (a mouse mastocytoma), and P815-S (P815 cells that stably express all the HBV Env protein (17). All of the cell lines were grown in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% FCS (Gemini Bioproduct, California), 4 nM l-glutamine (Irvin Scientific, Santa Ana, CA), 10 μg ml−1 of gentamicin (Irvin Scientific), and 5 × 10−5 mM 2-ME (Sigma). Target cells (3 × 105) were labeled with 300 μCi of 51Cr sodium chromate (NEN Research Products, Boston, MA) for 60 min at 37°C, washed three times, and resuspended in RPMI 1640/10% FCS at a concentration of 105 cells ml−1 in the absence or presence of 2 μg ml−1 of the appropriate peptides. To assay for CTL activity, 100 μl of target cells was incubated with 100 μl of different numbers of effector cells in U-bottom 96-well plates. Supernatants (100 μl) were removed after 4 h at 37°C and the percent lysis
was determined by the following formula: percent release = \( \frac{100 \times ([exponential~release} ~- ~spontaneous~release])}{(maximum~release ~- ~spontaneous~release]} \).

To allow averaging of different experiments, specific CTL activity was also expressed in LU (LU\(_{10^5}\)) in which 1 LU (LU\(_{10^5}\)) corresponds to the number of effector cells required to induce 30% lysis of 10\(^4\) \(^{51}\)Cr-labeled target cells during the 4-h assay (18). Thus, in the conditions used in the assay 1 LU\(_{10^5}\) represents 30% lysis at the 100:1 E:T ratio. Ten LU\(_{10^5}\) represents 30% lysis at the 1:1 ratio, 100 LU\(_{10^5}\) represents 30% lysis at the 1:1 ratio, and so on.

CTL activity was also measured by IFN-γ production. A total of 1 \(\times 10^6\) CTLs were cultured with 10\(^5\) target cells (HBV-Env-transfected or -untransfected P815 cells), with or without relevant peptide, in 96-well Immulon-2 plates (Dynex Technologies, Chantilly, VA) that were pre-coated with a capture anti-mouse IFN-γ Ab (clone R4-6A2; Pharmingen, San Diego, CA). After a 20-h incubation at 37°C, wells were washed with PBS/0.5% Tween 20 and incubated sequentially with a biotinylated secondary anti-mouse IFN-γ Ab (XMGI.2; Pharmingen), followed by streptavidin-HRP (Zymed, San Francisco, CA), and finally substrate (3,3',5,5'-tetramethylbenzidine + H\(_2\)O\(_2\); Pharmingen). Absorbance of each well was measured spectrophotometrically at 450 nm using an automated ELISA reader and the picograms per milliliter of IFN-γ captured in each well was determined by extrapolating from an IFN-γ standard curve.

**Injection of CTL lines**

Short-term CTL lines generated, as described above, were expanded in vitro in 6-well plates, each well containing 4 \(\times 10^6\) CTLs and 1.4 \(\times 10^7\) LPS/dextran-sulfate-activated splenocytes previously pulsed with 100 μg/ml peptide for 1 h at 37°C and irradiated with 3000 rad. Eighteen hours later, ConA-activated splenocyte supernatant (10% final concentration, 100 μg/ml) was added to cultures that were fed and expanded as necessary. Five days after the last stimulation, cells were then washed, counted, and suspended in PBS containing 2% FCS, and injected i.v. into recipient 1.3.32 HBV-transfected DBA/2 mice, with an average of 5.7 LU. Lower but still appreciable responses were noted in the case of Env.371 (not shown).

**Tissue DNA and RNA analyses**

Frozen liver tissue was mechanically pulverized under liquid nitrogen and total genomic DNA and RNA were isolated for Southern and Northern blot analyses exactly as previously described (17). Nylon membranes were analyzed for HBV DNA, HBV RNA, GAPDH and 2,5'-oligoadenylate synthetase as described elsewhere (19). Quantitation of cytotoxic T lymphocyte, T lymphocyte, and macrophage marker mRNAs was performed by RNase protection assay exactly as previously described (6, 19).

**Biochemical and histological analyses**

The extent of hepatocellular injury was monitored by measuring serum alanine aminotransferase (sALT) activity at multiple time points after treatment with saline or antigen. As expected, vigorous responses were observed in the case of Env.253 (recall CTL responses in 7/12 wild-type mice, with an average of 11.5 LU).

**Results**

**Identification of novel HBV-Env CTL epitopes**

Previous studies have characterized an HBV-Env-derived, H-2L\(^d\)-restricted dominant CTL epitope (17, 21, 22). To identify additional epitopes and thus being able to generate responses of maximum breadth, the HBV-Env protein sequence was scanned for the presence of H-2\(^d\) and H-2\(^b\) motif peptides (Table I). The corresponding peptides were tested for binding to purified MHC molecules in vitro. Consistent with previous studies (23, 24), a peptide was classified as a good binder when associated with an IC\(_{50}\) of 50 nM or less.

Two peptides that carried the K\(^b\) motif (Y\(_2\)L\(_{9,10}\)) (25) were identified, one of which, Env.362, bound with an IC\(_{50}\) of 16 nM. Of three peptides containing the D\(^b\) motif (G\(_P\)\(_{9,10}\)) (25), two (Env.364 and Env.281) bound with IC\(_{50}\) values of 5 and 115 nM, respectively. Of 20 peptides carrying the K\(^b\) motif (FY\(_{5}\)) (LIVM)\(_{8,9}\) (25), three (Env.329, Env.342, and Env.371) bound with IC\(_{50}\) values of 300, 150, and 469 nM, respectively. Finally, neither of the two D\(^b\) motif (N\(_2\)LIVM)\(_{8,9}\) (23, 24)-positive peptides bound with an IC\(_{50}\) of <500 nM. For the sake of comparison, the dominant L\(^d\)-restricted peptide was also tested for binding capacity and an IC\(_{50}\) of 2.4 nM was recorded.

Next, the immunogenicity of H-2\(^d\) and H-2\(^b\) class I binding peptides was evaluated in B6D2F \(_1\) mice. Analogous to previous studies (26, 27), peptides were emulsified inIFA in the presence of an excess (140 μg) of the I-A\(^\text{d,secreted}\)-helper epitope OVA.323–326. Herein, to allow averaging and combination in a single consistent format in several different experiments, specific CTL activity is expressed in LU (LU\(_{10^5}\)), in which 1 LU (LU\(_{10^5}\)) corresponds to the number of effector cells required to induce 30% lysis of 10\(^4\) \(^{51}\)Cr-labeled target cells during the 4-h assay (18). Thus, in the conditions used in the assay 1 LU\(_{10^5}\) represents 30% lysis at the 100:1 E:T ratio. Ten LU\(_{10^5}\) represent 30% lysis at the 1:1 ratio, 100 LU\(_{10^5}\) represent 30% lysis at the 1:1 ratio, and so on. Also, as defined previously (23, 24, 26), CTL responses of \(\geq 2\) LU\(_{10^5}/10^6\) cells were considered positive.

The results of testing the three newly identified H-2\(^b\)-binding peptides and the known dominant Env.28 epitope for immunogenicity are shown in Table II. As expected, vigorous responses were observed in the case of Env.28 (17/18 positive mice, 11.2 LU average response). Similar responses were observed in the case of Env.364, (12/14 mice yielded recall CTL responses, with an average of 13.6 LU). Lower but still appreciable responses were noted in the case of Env.362 (recall CTL responses in 7/12 wild-type mice, with an average of 5.7 LU). Finally, in the case of Env.281, recall CTL responses were observed in only 2 of 10 mice tested (6.2 LU average magnitude).

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**Table 1. In vitro binding capacity of HBV-Env-derived peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Motif</th>
<th>Binding Capacity (IC(_{50}), nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Env.362</td>
<td>WYWGPSLYSI</td>
<td>K(^d)</td>
<td>16</td>
</tr>
<tr>
<td>Env.368</td>
<td>LYSLISFLP</td>
<td>K(^d)</td>
<td>2500</td>
</tr>
<tr>
<td>Env.364</td>
<td>WGPSLYSIL</td>
<td>D(^b)</td>
<td>5.0</td>
</tr>
<tr>
<td>Env.281</td>
<td>TGPCRTCMT</td>
<td>D(^b)</td>
<td>115</td>
</tr>
<tr>
<td>Env.281</td>
<td>TGPCRTCMTT</td>
<td>D(^b)</td>
<td>4286</td>
</tr>
<tr>
<td>Env.28</td>
<td>IPSLDSSWWTSSL</td>
<td>L(^d)</td>
<td>2.4</td>
</tr>
<tr>
<td>Env.150</td>
<td>LSIFSIR</td>
<td>K(^b)</td>
<td>3333</td>
</tr>
<tr>
<td>Env.155</td>
<td>SRIGDPAL</td>
<td>K(^b)</td>
<td>—*</td>
</tr>
<tr>
<td>Env.165</td>
<td>ENITSGFL</td>
<td>K(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.178</td>
<td>LQAQFFL</td>
<td>K(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.231</td>
<td>TCPGTYRWM</td>
<td>K(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.233</td>
<td>PGYRWNL</td>
<td>K(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.243</td>
<td>RFIILFI</td>
<td>K(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.243</td>
<td>FIIFILFIL</td>
<td>K(^b)</td>
<td>5000</td>
</tr>
<tr>
<td>Env.244</td>
<td>IIIFILFIL</td>
<td>K(^b)</td>
<td>7500</td>
</tr>
<tr>
<td>Env.247</td>
<td>LFIILILCL</td>
<td>K(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.252</td>
<td>LCLIFILLV</td>
<td>K(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.253</td>
<td>CLIFILVL</td>
<td>K(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.259</td>
<td>VLLDQYQGM</td>
<td>K(^b)</td>
<td>3333</td>
</tr>
<tr>
<td>Env.329</td>
<td>ASARFSWL</td>
<td>K(^b)</td>
<td>300</td>
</tr>
<tr>
<td>Env.342</td>
<td>FQVFVFGL</td>
<td>K(^b)</td>
<td>150</td>
</tr>
<tr>
<td>Env.361</td>
<td>MWYWGPSL</td>
<td>K(^b)</td>
<td>7500</td>
</tr>
<tr>
<td>Env.365</td>
<td>GPSLYSIL</td>
<td>K(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.369</td>
<td>YISILSPFL</td>
<td>K(^b)</td>
<td>1111</td>
</tr>
<tr>
<td>Env.371</td>
<td>ILSPFLL</td>
<td>K(^b)</td>
<td>469</td>
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<tr>
<td>Env.378</td>
<td>LLDIRFCL</td>
<td>K(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.159</td>
<td>DPALMNENI</td>
<td>D(^b)</td>
<td>—</td>
</tr>
<tr>
<td>Env.305</td>
<td>PSDGNCTCI</td>
<td>D(^b)</td>
<td>—</td>
</tr>
</tbody>
</table>

*Dash indicates \(\geq 10,000\).*
Of the three HBV-ENV-derived H-2b binders, only one (Env.371) yielded a relatively weak CTL-positive response (3.0 LU), and only in two of the six mice tested. Two control H-2Kb-restricted class I epitopes (lymphocytic choriomeningitis virus (LCMV) Np.396 and OVA.257) yielded vigorous responses (average magnitudes of 6.5 and 16.9 LU, respectively) with 100% of the animals responding (data not shown).

Recognition of naturally processed HBV-ENV Ag by CTLs specific for the Env.362 and Env.364 epitopes

To determine whether the Env.362 and the Env.364 epitopes were generated in the course of natural processing of the HBV-Env Ag, short-term CTL lines were derived by peptide immunization of nontransgenic (B6D2F1) mice and tested for their capacity to recognize P815-S target cells (17).

As shown in Fig. 1, CTLs specific for control epitopes were completely devoid of cytotoxicity for the HBV-Env-transfected P815 target cells in multiple independent experiments (Fig. 1, a and b). As expected, high levels of specific killing were detected in the case of Env.28 (Fig. 1c), with 51Cr release approaching 100% at an E:T of 10:1. Recognition of P815-S target cells was also detected in the case of both Env.364- and Env.362-specific CTL lines (Fig. 1, d and e) with 51Cr release approaching 50% at an E:T of 10:1 for both cultures.

It should be noted that in the case of Env.364 and Env.362, recognition of target cells expressing naturally processed Ag was relatively less efficient (as compared with peptide-pulsed targets) for these two epitopes (as compared with the dominant Env.28 epitope), perhaps signifying that these epitopes are less abundantly produced by natural processing, which might in turn explain their subdominant status.

Immunogenicity of HBV-Env-derived H-2d-binding peptides

Table II. Immunogenicity of HBV-Env-derived H-2d-binding peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>No. positive/No. tested</th>
<th>LU 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Env. 28</td>
<td>17/18</td>
<td>11.2 (2.4)*</td>
</tr>
<tr>
<td>Env. 364</td>
<td>12/14</td>
<td>13.6 (2.0)</td>
</tr>
<tr>
<td>Env. 362</td>
<td>7/12</td>
<td>5.7 (1.5)</td>
</tr>
<tr>
<td>Env. 281</td>
<td>2/10</td>
<td>6.2 (1.5)</td>
</tr>
</tbody>
</table>

*Peptides were dissolved in DMSO/PBS. An IFA emulsion containing 50 μg of each CTL epitope and 140 μg of OVA.323 helper epitope was injected in B6D2F1 mice. Eleven days later, splenocyte cultures were stimulated with 10 μg/ml of specific CTL epitopes and 107 syngeneic irradiated LPS blast cells. After 6 days, chromium release CTL assays were performed using 51Cr-labeled P815 (Kb) target cells in the presence or absence of specific CTL epitopes.

**Geometric mean of positive cultures (X/± SD).**

FIGURE 1. Recognition of naturally processed HBV-Env epitopes by short-term CTL lines. Groups of three individual B6D2F1 mice were injected with 50 μg of indicated CTL epitopes (a, LIS.91; b, LCMV Np.118; c, Env. 28; d, Env.364; and e, Env.362) and 140 μg of the OVA.323 HTL epitope emulsified in IFA. Eleven days later, spleens were removed and pooled. Splenocytes were stimulated with 10 μg/ml of each specific CTL epitope. After 10 days, cultures were restimulated using syngeneic irradiated LPS blast cells pulsed with 10 μg/ml of each specific CTL epitope. Cultures were assayed 5 days later for cytolytic activity using a standard 4-h 51Cr release assay using P815 target cells in the presence (●) or absence (△) of the specific peptide or PreS1-transfected P815 target cells (□).
to those induced in nontransgenic mice. These results demonstrate that T cell tolerance can be overcome by lipopeptide immunization at the level of induction of recall CTL activity. However, unlike the CB6F1-derived CTLs, the CTLs derived from the HBV-transgenic mice did not recognize endogenously synthesized Ag since they failed to kill HBV-Env-transfected target cells. Therefore, chromium release CTL assays were performed using 31Cr-labeled P815 (K+)-target cells in the presence or absence of specific CTL epitopes or P815-S target cells.

Immunogenicity of a lipopeptide pool in nontransgenic and 1.3.32 HBV-transgenic mice: recognition of peptide-pulsed target cells

**Table III. Immunogenicity of a lipopeptide pool in nontransgenic and 1.3.32 HBV-transgenic mice: recognition of peptide-pulsed target cells**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>CTL Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nontransgenic</td>
</tr>
<tr>
<td>No. positive/No. tested</td>
<td>LU30</td>
</tr>
<tr>
<td>Env. 28</td>
<td>3/3</td>
</tr>
<tr>
<td>Env. 364</td>
<td>3/3</td>
</tr>
<tr>
<td>Env. 362</td>
<td>3/3</td>
</tr>
</tbody>
</table>

*a* Lipopeptides were formulated as described in Materials and Methods and injected as a pool into nontransgenic CB6F1, or 1.3.32 HBV CB6F1-transgenic mice. Eleven days later, splenocyte cultures were stimulated with 10 μg/ml of specific CTL epitopes and 10^7 syngeneic irradiated LPS blast cells. After 6 days, chromium release CTL assays were performed using 31Cr-labeled P815 (K+)-target cells in the presence or absence of specific CTL epitopes or P815-S target cells.

In the next series of experiments, we compared the avidity of the CTLs generated in nontransgenic CB6F1 mice and in 1.3.32 lineage CB6F1 mice using a dose titration of the various epitopes. One of two independent experiments yielding similar results is shown in Fig. 2. In the case of the Env.28 epitope, the concentration of peptide necessary to achieve 30% lysis by CTLs from 1.3.32 CB6F1-transgenic mice and nontransgenic mice was 5.9 and 0.13 pg/ml, respectively. In the case of the Env.364 epitope, the concentrations necessary for 30% lysis were 276 and 0.256 ng/ml for 1.3.32 and CB6F1-derived CTL lines, respectively. Thus, in both cases, CTL lines derived from transgenic mice displayed 100- to 1000-fold lower avidity for their targets. In contrast, little or no difference in avidity was detected in the case of the Env.362 epitope.

**Transfer of CTL derived from 1.3.32-transgenic mice is associated with HBV Ag down-regulation in the absence of liver pathology**

Adoptive CTL transfer experiments were performed next to further characterize the quality of the recall CTL activity elicited by lipopeptide immunization in the transgenic and nontransgenic mice. Groups of three CB6F1 mice were immunized with a pool of the three lipided PADRE-CTL constructs encompassing the Env.28, 362, and 364 CTL epitopes. After 1 mo, all groups were boosted with the same immunogens. Eleven to 14 days later, splenocytes were harvested and CTL-specific lines were expanded in vitro with the appropriate CTL epitopes.

Five days after the second in vitro stimulation, CTL cultures from each group were tested for activity in a 31Cr release assay. Data from one representative experiment is shown in Table V. Cultures derived from normal CB6F1 mice yielded strong CTL activity in the 60–220 LU range, and cultures derived from 1.3.32-transgenic mice yielded slightly less CTL activity (in the 40–160 LU range) against P815 target cells pulsed with the various HBV-Env-derived epitopes.

Equal numbers of the nontransgenic or transgenic CTL lines specific for the three different HBV-Env CTL epitopes were pooled, and a total of 1 × 10^7 nontransgenic or transgenic cells were injected in groups (six mice per group) of 8- to 10-wk-old females and serum hepatitis B e Ag-matched animals from lineage 1.3.32 (CB6F1, hybrids) HBV-transgenic mice. An equal number of cells from CTLs specific for the LCMV Np.118 CTL epitope was also injected into the HBV-transgenic mice to control for nonspecific effects.

Transfer of nontransgenic HBV-specific CTL lines into transgenic mice was associated with increased levels of sALT activity (Table VI), and the presence of small, scattered necroinflammatory foci in the liver parenchyma (data not shown), both at day 1 and day 3 after transfer (data not shown). As shown in Fig. 3, 1 day after injection, HBV DNA replication was strongly inhibited in the liver of these animals when compared with saline-injected controls. This was associated with the intrahepatic production of IFN-γ, TNF-α, CD8, CD4, CD3, and F480 mRNA (Fig. 3). These results are not surprising since using the same mouse model we have previously shown that the antiviral potential of the CTLs is primarily mediated by noncytolytic mechanisms that involve the intrahepatic production of IFN-γ (6, 7).

Transfer of transgenic HBV-Env-specific CTLs into transgenic mice was not associated with increased sALT activity and very few necroinflammatory foci were detected in the liver (data not shown). The signal for CD8, CD4, CD3, and F480 RNAs (which indicates the number of liver-infiltrating inflammatory cells, including the adoptively transferred CTLs) in these animals was higher than that of saline-injected controls and lower than that of mice that received nontransgenic CTLs (Fig. 3). This is consistent with their lower avidity (except Env 362) and poorer recognition of endogenously synthesized Ag than the nontransgenic CB6F1, CTLs. Nonetheless, HBV replication was strongly inhibited in the animals that received transgenic CTLs, almost to the same degree observed in the mice that received nontransgenic CTLs. This was
associated with a moderate induction of IFN-γ, indicating that Ag recognition has occurred in vivo (Fig. 3). Finally, no liver disease was detected in the control group that received LCMV-specific CTLs. As expected, HBV replication was not affected and IFN-γ production was not induced in the liver of these mice (Fig. 3).

These in vivo observations were paralleled by in vitro experiments which demonstrated that CTL derived from HBV-transgenic mice did not kill HBV-Env-transfected target cells, but were capable of secreting IFN-γ, albeit in small amounts, when confronted with HBV-Env-transfected targets. Control CTL lines derived from normal mice were capable, by contrast, of both killing and IFN-γ secretion in response to HBV-Env-transfected p815 target cells (Fig. 4).

Discussion

Immunogenicity screening in nontransgenic mice of H-2d-binding, HBV-Env-derived CTL peptides, emulsified in IFA, revealed two new subdominant CTL epitopes (Env.362 and Env.364) which elicited CTL specificities that recognized naturally processed Ag in the form of HBV-Env-transfected target cells. In contrast, only three H-2b-binding peptides were identified, and they bound with relatively low affinity and were found to be essentially nonimmunogenic. These results are consistent with the observation that H-2b mice are CTL nonresponders for the Env protein (28, 29).

Relative to Env.28, recognition of endogenously synthesized Ag was less efficient in the case of the subdominant epitopes (Env.364 and Env.362), suggesting that these epitopes are produced less efficiently by natural HBV-Env processing. This, along with their lower binding affinity relative to Env.28, might explain their subdominant status (30). Analysis of lineage 1.3.32 HBV-transgenic mice, in terms of CTL responsiveness directed against the new H-2d-restricted epitopes and the previously defined dominant Env.28 epitope, demonstrated T cell hyporesponsiveness in 1.3.32 mice for the Env.28 and Env.364, but less so in the case of the Env.362 epitope. It is possible that this subdominant epitope might be a less effective tolerogen because it is naturally expressed in lower amounts in hepatocytes of 1.3.32 HBV-transgenic mice. These results suggest that targeting subdominant epitopes may be an effective way to overcome CTL tolerance.

Studies in nontransgenic H-2d mice demonstrated that a mixture of HTL-CTL epitope lipoconstructs was capable of simultaneously inducing responses for each of these CTL epitopes. In 1.3.32 HBV-transgenic mice, this immunization strategy was also effective in inducing recall CTL activity. These results are significant because previous attempts aimed at overcoming CTL tolerance in this lineage of transgenic mice had not met with success (8). A crucial element of this strategy appears to be the use of preprocessed epitopes, as in this system whole HBV-Env DNA immunization does not break CTL tolerance (8), but epitope-based minigenes do (A. D. Sette, C. Oseroff, and J. Alexander, unpublished observations). However, the induction of HBV-specific CTLs was not associated with either down-regulation of expression of HBV Ags in the liver or with increased sALT activity, indicating lack of hepatocyte damage. Thus, the induced CTLs either did not reach the liver in sufficient numbers to exert detectable effector functions, or the CTLs were functionally defective in the transgenic mice, or the target Ags were not efficiently processed. The last explanation is unlikely since all three epitopes are presented by Env-transfected target cells (Fig. 1) and because Env.28 CTLs are

Table V. Activity of CTL lines used for adoptive transfer experiments

<table>
<thead>
<tr>
<th>Immunization</th>
<th>In Vitro Restimulation</th>
<th>CTL Activity a (LU 30 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool of HBV-Env CTL lipopeptides</td>
<td>Env. 28</td>
<td>216.5 (1.05)</td>
</tr>
<tr>
<td></td>
<td>Env. 364</td>
<td>71.8 (1.1)</td>
</tr>
<tr>
<td></td>
<td>Env. 362</td>
<td>64.7 (1.2)</td>
</tr>
<tr>
<td>LCMV Np.118 lipopeptide</td>
<td>LCMV Np.118</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Nontransgenic CB6F1 or 1.3.32 HBV-transgenic CB6F1 mice were injected and boosted with a pool of lipopeptides encoding the three HBV-Env epitopes as described in Materials and Methods. Splenocytes were restimulated twice in vitro with each epitope separately and then tested for activity in a standard chromium release assay.

b Geometric mean (X±SD) of the lytic activity for peptide-pulsed P815 target cells of three different cultures.
known to recognize their target epitopes very efficiently in vivo (6, 20, 21).

Direct visualization and/or detection of CTL activity in freshly harvested splenocytes by means of tetrameric staining was not attempted in the current set of experiments. Future studies might investigate the relationship between these CTLs and the functionally impaired CTLs observed in the course of chronic infection with various viruses including LCMV, hepatitis C virus, and HBV (2, 31, 32). It is important to point out, however, that in these cases, functionally impaired CTLs were associated with chronic exposure to viral Ags, whereas in our cases, these CTLs are induced de novo after immunization of animals already chronically expressing large amounts of HBV Ags.

Further adoptive transfer studies characterized the CTLs induced in 1.3.32-transgenic mice in more detail. It was found that these CTLs were effective in inhibiting viral replication. However, unlike the control CTLs induced in nontransgenic mice, they did not cause hepatitis. This antiviral effect is likely mediated by noncytolytic mechanisms that involve intrahepatic production of IFN-$\gamma$ (6, 33). Peptide titration experiments demonstrated that CTLs derived from 1.3.32 HBV-transgenic mice were characterized by low avidity, thus suggesting a possible mechanism to account for these observations if the lower avidity recognition can trigger the release of antiviral cytokines without significantly activating the cytolytic function of the CTLs. The CTLs induced in nontransgenic mice were more effective than those induced in nontransgenic mice, possibly a reflection of both cytolytic and noncytolytic mechanisms as well as potentially higher levels of production of noncytolytic mediators. Nevertheless, it is obvious that selective induction of antiviral effects in chronically infected individuals, in the absence of tissue damage, would be of significant therapeutic interest.

These results are also consistent with previous observations by Sherman and coworkers (34) and von Herrath et al. (35) and demonstrated that low-avidity CTLs can escape tolerance inactivation and might be stimulated by immunization with vaccine.

### Table VI

Transfer of 1.3.32-derived CTL lines is not associated with increased sALT levels in 1.3.32 HBV-transgenic recipients

<table>
<thead>
<tr>
<th>Specificity and Origin of CTL Line Transferred</th>
<th>Mouse</th>
<th>Day After Transfer</th>
<th>1 (U/ml)</th>
<th>2 (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV-specific, 1.3.32 transgenic</td>
<td>1</td>
<td>51</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>51</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>HBV-specific, H-2-matched control</td>
<td>1</td>
<td>174</td>
<td>348</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>258</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>LCMV-specific, 1.3.32 transgenic</td>
<td>1</td>
<td>63</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>48</td>
<td>69</td>
<td></td>
</tr>
</tbody>
</table>

$sALT$ levels in two control 1.3.32 HBV-transgenic mice that did not receive any cell transfer were 54 and 66 U/L.

FIGURE 3. Transfer of CTL derived from 1.3.32-transgenic mice inhibits HBV replication. Age-matched, sex-matched, and serum hepatitis B e Ag-matched lineage 1.3.32 HBV-transgenic mice were injected i.v. with $1 \times 10^7$ nontransgenic or transgenic CTLs and sacrificed 24 h later. Control mice were injected with either saline (NaCl) or CTL derived from nontransgenic mice immunized with an LCMV-specific peptide. Total hepatic DNA was analyzed for HBV DNA by Southern blot analysis. All DNA samples were RNase treated before quantitation and gel electrophoresis. Bands corresponding to the integrated transgene, relaxed circular (RC) double-stranded HBV DNA, and single-stranded (SS) linear HBV DNA replicative forms are indicated. The integrated transgene can be used to normalize the amount of DNA bound to the membrane. The filter was hybridized with a $^{32}$P-labeled HBV-specific DNA probe. Total hepatic RNA was analyzed for cytokine, T cell, and macrophage marker transcripts by RNase protection assay, as indicated. The mRNA encoding the ribosomal protein L32 was used to normalize the amount of RNA loaded in each lane of the RNase protection assay.

FIGURE 4. IFN-$\gamma$ secretion and killing activity of CTL lines derived from 1.3.32-transgenic mice. The capacity of short-term CTL lines generated from 1.3.32-transgenic and -nontransgenic mice against three Env epitopes to display epitope-specific cytotoxic activity or IFN-$\gamma$ production was determined. CTLs were tested for effector function against P815 tumor cells in the presence or absence of peptide, or against P815 cells transfected with the HBV Env gene. Cytotoxic activity was measured using the standard $^{51}$Cr release assay at a 10:1 E:T ratio while cytokine production was determined using an in situ capture ELISA. Net CTL responses are shown after subtracting background. CTL activity against P815 cells in the absence of exogenous peptide (background) ranged from 4 to 16% (cytotoxicity) and 17 to 96 pg/well (IFN-$\gamma$).
constructs using preprocessed optimal epitopes. A crucial point, which remains to be addressed, is why, in the current study, induction of this CTL specificity did not affect viral replication in the immunized animals, but only in the adoptive transfer experiments. Our results are compatible with two nonmutually exclusive explanations. First, it is possible that in vitro expansion might in some way reverse a functional inactivation state. Alternatively, and in our view more likely, it is possible that the magnitude, in terms of the number and expansion of CTL specificities induced in the transgenic mice, is suboptimal and in vitro expansion is necessary to achieve sufficient numbers of CTL effectors.

In this light, we are currently investigating different immunization protocols such as prime/boost strategies (36) and the combined use of multiple epitopes from various HBV Ags as a way to optimize the magnitude and breadth of CTL responses. Other studies have also emphasized the importance of optimized helper activity in overcoming CTL unresponsiveness (37). Taken together, we believe that these results are very encouraging in terms of potential immunotherapy of HBV infection in humans in which, as a result of the recent availability of antiviral compounds such as lamivudine, problems related to viral expression and its suppressive effects might be less severe.

In conclusion, we have shown, for the first time, that immunization of HBV 1,3,3,2-transgenic mice can result in induction of CTL activity capable, in passive transfer experiments, of downregulating expression of viral Ags in the absence of hepatocyte damage. These observations have obvious implications for development of therapeutic strategies to treat chronic HBV infection.

Finally, we would also like to point out that these results presented in this manuscript may also have implications for autoimmune disease and cancer treatments. In several instances, it has been reported that T cells involved in autoimmune phenomena (38, 39) or directed against tumor-associated Ags (34) are associated with altered avidity or patterns of cytokine release. Thus, understanding the structural basis of the cytotoxic mechanisms described herein may reveal interesting avenues for treatment of several complex diseases.

Acknowledgments

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References


