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Distinct, Cross-Reactive Epitope Specificities of CD8 T Cell Responses Are Induced by Natural Hepatitis B Surface Antigen Variants of Different Hepatitis B Virus Genotypes

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We investigated the specific and cross-reactive CD8 T cell immunity to three natural variants (of different geno/serotype) of the small hepatitis B surface Ag (or S protein). The Dd-binding variants of the S$_{201-209}$ epitope showed different immunogenicity. The loss of the consensus C-terminal (P9) anchor abrogated its immunogenicity. In contrast, a conservative (serine vs asparagine) exchange at P7 primed cross-reactive CD8 T cells that preferentially recognized the priming variant. Cross-reactive CD8 T cell responses to a variant could be primed in mice tolerant to an alternative variant of the Dd-binding S$_{201-209}$ peptide. Loss of the C-terminal (P10) anchor in S$_{185–194}$ eliminated its immunogenicity in HLA-A*0201(A2)-transgenic mice but two conservative exchanges (leucine vs valine in P2, and leucine vs isoleucine in P6) in S$_{208–216}$ generated cross-reactive CD8 T cell responses with strong preference for the priming variant. Similar cross-reactive recognition of variant envelope epitopes were also found in S$_{208–216}$-specific CD8 T cells from hepatitis B virus (HBV)-infected patients. Distinct CD8 T cell populations cross-reactive to natural variants of class I-restricted HBV epitopes can be primed by vaccination (of mice) or natural infection (of humans), and they may play a role in the “spontaneous remission” or the specific immunotherapy of chronic HBV infection. The Journal of Immunology, 2006, 176: 4003–4011.

The hepatitis B virus (HBV)$^3$ has a small, very compact genome organization, an unusually low mutation fixation rate, and a limited number of genotypes and serotypes (1, 2). The virus chronically infects ~350 million people worldwide and is a major cause of liver disease and hepatocellular carcinoma. Current antiviral treatments are disappointing, as only 30% of treated patients are able to maintain stable control of HBV replication (3). Because T cells play a major role in controlling HBV infection (4), different vaccine formulations have been used in chronically infected patients in an attempt to elicit HBV-specific T cell responses that can control HBV replication. However, therapeutic vaccinations have been so far disappointing. This may be due to the preferential use of vaccination procedures designed to elicit a prophylactic humoral response in healthy subjects (5). Furthermore, fundamental characteristics of the HBV-specific T cell response present during chronic HBV infection (6–8) may limit vaccination efficacy as HBV-specific CD4 and CD8 T cells are mostly deleted or functionally impaired in hepatitis B patients with high levels of viremia.

An alternative strategy to elicit HBV-specific T cell responses in patients with chronic hepatitis may be the use of variant epitopes of HBV to induce “new” specific T cell responses that cross-react with epitopes derived from the infecting virus. Natural variability within HBV proteins exists, particularly among the different HBV isolates from the genotypes A–G (with >8% divergence between them) (9, 10). These genotypes have diverse geographical distributions, and their variability seems to influence the clinical course of the disease (11, 12). Sequence differences between HBV genotypes may lead to differences in virus replication and translation but can also result in differences in the immunogenicity of the viral proteins, particularly for T cells. We previously reported (13, 14) that CD8 T cell responses to natural variants of a Kb-restricted epitope of the hepatitis B surface Ag (HBsAg) can specifically break tolerance in HBsAg-transgenic mice. In this study, we follow up on these studies by analyzing murine cross-reactive CD8 T cell responses to variants of Dd$^2$ and HLA-A$^*0201$-restricted HBsAg epitopes and human CD8 T cell responses to variants of the HLA-A$^*0201$-restricted S$_{208–216}$ epitope of HBsAg.

We focus on the HBV small envelope protein (HBsAg) in this study (15). MHC class I-binding determinants that specifically stimulate CD8 T cells from humans and mice have been identified in the small HBsAg protein. The natural sequence variability of HBV Ags is 5–10% (10, 16). This limited variability (15–18 residue exchanges in a 226-aa protein) allows us to analyze differences in the immunogenicity and cross-reactivity between MHC class I-binding determinants with single residue exchanges. In this study, we used nontransgenic and HBsAg-transgenic mice to study the specific and cross-reactive CD8 T cell responses to natural variants of the Dd$^2$-binding S$_{201–209}$ epitope of the HBsAg. In addition, we identified cross-reactive CD8 T cells from HLA-A$^*0201$-transgenic mice that recognize natural variants of the HLA-A$^*0201$-binding S$_{185–194}$ and S$_{208–216}$ epitopes. Finally, we asked whether CD8 T cells preferentially stimulated by particular
natural variants of the HLA-A*0201-binding S<sub>208–216</sub> epitope are found during natural HBV infection in patients.

Materials and Methods

**Mice**

L<sup>2</sup>– H<sup>2</sup><sup>m2</sup> BALB/c (dm2) mice, HLA-A*0201-transgenic HHB (A2-tg) mice (17), H<sup>2</sup>– C57BL/6Bom (B6) mice, (dm2 × B6)<sub>F1</sub> mice, C57BL/6<sub>1</sub>-Tg(NAlbHBV)4Bri-transgenic (pAlb-HBs) mouse (18), and (dm2 × pAlb-HBs)<sub>F2</sub> mice were bred and kept under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). Male and female mice were used at 12–16 wk of age.

**Cells**

The H<sup>2</sup>– mastocytoma cell line P815 was obtained from the American Type Culture Collection (TIB64). The human lymphoblastoid cell line C1R and the stable K<sub>d</sub>- and D<sub>d</sub>-expressing transfectants derived from this line were a generous gift from Dr. P. van Endert (Institut National de la Sante et de la Recherche Medicale J580, Paris, France).

**HBsAg vaccines**

We selected for this study three sequences encoding the complete, small HBsAg protein of genotype A, C, and D listed in Table II. These sequences were cloned into the pCI expression vector (catalog no. E1731; Promega) to generate the expression plasmids pCI/S-A, pCI/S-C, and pCI/S-D. All three vectors expressed comparable amounts of HBsAg after transient transfection of these DNAs into LMH cells (13).

**Immunization of mice**

Intramuscular nucleic acid immunization was achieved by injecting 50 μg (1 μg/μl) plasmid DNA into each tibialis muscle (i.e., 100 μg plasmid DNA/mouse) as described previously (20). Mice were usually vaccinated only once. Where indicated, mice were boosted twice at days 28 and 52 after priming.

**Synthetic peptides**

The synthetic, HBsAg variant-specific peptides used are listed in Table II and were obtained from JPT Peptide Technologies. Peptides were dissolved in DMSO at a concentration of 1–10 mg/ml and diluted with culture medium before use.

**Determination of the frequency of specific CD8 T cells**

Spleen cells (1 × 10<sup>7</sup> ml) were incubated for 1 h in RPMI 1640 medium either with the indicated amounts of the HBsAg peptides or with C1R/D<sup>d</sup> cells prepulsed with the indicated amounts of the HBsAg peptides (10<sup>6</sup> ml). Thereafter, brefeldin A (catalog no. 15870; Sigma-Aldrich) was added to a final concentration of 5 μg/ml and the cultures were incubated for another 4 h. Cells were harvested, washed, and surface stained with PE-conjugated anti-CD8 mAb (catalog no. 01045B; BD Pharmingen). Surface-stained cells were fixed with 2% paraformaldehyde in PBS. Fixed cells were re-suspended in permeabilization buffer (HBSS, 0.5% BSA, 0.5% saponin, and 0.05% sodium azide) and incubated with FITC-conjugated anti-IFN-γ mAb (catalog no. 55441; BD Pharmingen) or FITC-conjugated anti-TNF-α mAb (catalog no. 554418; BD Pharmingen) for 30 min at room temperature and washed twice in permeabilization buffer. Stained cells were re-suspended in PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide. We determined the frequencies of CD<sup>8</sup> IFN-γ<sup>+</sup> T cells by flow cytometry analyses. The mean number of CD<sup>8</sup> IFN-γ<sup>+</sup> T cells or CD<sup>8</sup> TNF-α<sup>+</sup> T cells per 10<sup>6</sup> CD<sup>8</sup> spleen cells of three to five individual mice (±SEM) is shown.

**IFN-γ detection by ELISA**

IFN-γ was measured in cell culture supernatants by double-sandwich ELISA (catalog no. 55082; BD Pharmingen).

**Chromium release assays**

Single-cell suspensions were prepared from spleens of mice in Click's RPMI 1640 tissue culture medium supplemented with 10 mM HEPES buffer, 5 × 10<sup>–5</sup> M 2-ME, antibiotics, and 10% v/v FCS (PAA Laboratories). A selected batch of Con A-stimulated rat spleen cell supernatant (2% v/v) was added to the culture medium. Three × 10<sup>5</sup> responder cells were cocultured with 1 × 10<sup>6</sup> irradiated P815 cells prepuled for 4 h with 100 μg/ml of the relevant HLA class I-binding peptide. The coculture was performed in 10 ml of medium in upright 25-cm<sup>2</sup> tissue culture flasks in a humidified atmosphere/5% CO<sub>2</sub> at 37°C. After 5 days of culture, CTL were harvested, washed, and assayed for specific cytolytic reactivity. Serial dilutions of effector cells were cultured with 2 × 10<sup>5</sup> 51-Cr-labeled targets in 200-μl round-bottom wells. Specific cytolytic activity of cells was tested in short-term 51-Cr release assays against HBsAg-expressing or nonexpressing P815 transfectants. After a 4-h incubation at 37°C, 50 μl of supernatant was collected for gamma radiation counting. The percentage of specific release was calculated as (experimental release – spontaneous release)/ (total release – spontaneous release) × 100. Total counts were measured by resuspending target cells. Spontaneously released counts were always <15% of the total counts. Data shown are the mean of triplicate cultures. The SEM of triplicate data was always <20% of the mean.

**Patients**

Eighteen HLA-A2-positive adult subjects who resolved acute hepatitis B infection were studied. All subjects were anti-HBc and anti-HBs Ab positive and negative for Abs to hepatitis C virus, δ-virus, or HIV-1,2.

**In vitro expansion of human HBsAg-specific CD8 cells**

PBMC were placed in 96-well plates at 2 × 10<sup>5</sup> ml and stimulated with HBV peptides at 1 μM final concentration. rIL-2 was added on day 4 of culture (20 U/ml). Cell lines were tested on day 10 by immunological assays (IFN-γ production and CD107 expression) to detect peptide-specific CD8 T cells or further expanded for selection of S<sub>208–216</sub>-specific CTL clones. Briefly, the IFN-γ capture assay (Milenyi Biotec) was used to select peptide-specific CD8 T cells. Selected cells were seeded at different concentrations (1, 5, and 10 cell/well) in 96-well plates with 10<sup>5</sup> irradiated (3000 rad) allogeneic PBMC and PHA (0.5 μg/ml)/well. Cells were re-stimulated every 2 wk with allogeneic-irradiated PBMC, and growing cell lines were expanded with A-AIM medium (Invitrogen Life Technologies) supplemented with IL-2 (20 U/ml), IL-7 (10 ng/ml), and IL-15 (10 ng/ml). Lines were tested for peptide specificity, and cell lines showing >90% of peptide-specific CD8 cells were maintained and further expanded.

**CD107 assay**

PBMC-derived lines were stimulated with indicated concentrations of peptides in the presence of FITC-labeled anti-CD107 Abs (BD Pharmingen) (21). After 4 h of stimulation at 37°C in a CO<sub>2</sub> incubator, cells were harvested once with PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide, resuspended in supplemented PBS and PE-labeled anti-CD8

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**Table I. Variants of the HBV (HBsAg) used**

<table>
<thead>
<tr>
<th>HBsAg Genotype Variants</th>
<th>Serotype</th>
<th>Swiss Prot code (primary accession no.)</th>
<th>DNA Vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>adw</td>
<td>VMSA.HPBV2 (P03141)</td>
<td>pCI/S-A</td>
</tr>
<tr>
<td>C</td>
<td>adr</td>
<td>VMSA.HPBVR (P03140)</td>
<td>pCI/S-C</td>
</tr>
<tr>
<td>D</td>
<td>ayw</td>
<td>VMSA.HPBVY (P03138)</td>
<td>pCI/S-D</td>
</tr>
</tbody>
</table>

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**Table II. The CD8 T cell-defined, variant HBsAg determinants studied**

<table>
<thead>
<tr>
<th>HBsAg Residue</th>
<th>Restriction</th>
<th>Variant A</th>
<th>Variant C</th>
<th>Variant D</th>
</tr>
</thead>
<tbody>
<tr>
<td>201–209</td>
<td>D&lt;sup&gt;a&lt;/sup&gt;</td>
<td>wGPSLYSTV</td>
<td>wGPSLYNL</td>
<td>wGPSLYSTL</td>
</tr>
<tr>
<td>185–194</td>
<td>HLA-A* 0201</td>
<td>aLSPTVWLSA</td>
<td>aLSPTVWLSY</td>
<td>aLSPTVWLSY</td>
</tr>
<tr>
<td>208–216</td>
<td>HLA-A* 0201</td>
<td>iVSPFPiPLL</td>
<td>iLSPPF&lt;sub&gt;2&lt;/sub&gt;iPL</td>
<td>iLSPPF&lt;sub&gt;2&lt;/sub&gt;iPL</td>
</tr>
</tbody>
</table>

<sup>a</sup> Anchors in bold; variant residues underlined.
Ab added to the wells. After 20 min of incubation at 4°C, cells were washed twice. The frequency of CD8⁺CD107⁺ T cells was analyzed by flow cytometry analyses.

**Results**

**Murine cross-reactive CD8 T cell responses are elicited by some but not all natural variants of the Dd-restricted S201–209 determinant of HBsAg**

We cloned the complete sequences of three natural variants of the small HBsAg of the HBV genotype A, C, and D (Table I) into the expression plasmid pCI to construct the DNA vaccines pCI/S-A, pCI/S-C, and pCI/S-D. Ld-deficient dm2 mice were immunized with the pCI/S-A, pCI/S-C, or pCI/S-D DNA vaccines. dm2 mice were used to eliminate suppression of CD8 T cell responses to the Dd-restricted S201–209 epitope by CD8 T cells specific for the dominant, Ld-restricted S28–39 epitope (22–24).

Variant S201–209 peptides C and D have consensus anchor residues at P2, P3, and P9 but differ at P7 where an asparagine (N) is exchanged for a serine (S) (Table II). Vaccination of dm2 mice with pCI/S-D- or pCI/S-D-primed CD8 T cells that preferentially recognized the priming epitope, but also cross-reacted with the D or C variant, respectively (Fig. 1). Variant A of the S201–209 epitope has a valine (V) at the anchor P9 residue where variant D has the consensus leucine (L) residue (25, 26) (Table II). dm2 mice vaccinated with pCI/S-A did not develop CD8 T cells specific for the priming epitope (Fig. 2A). Variant A-specific CD8 T cells could not be detected after repeated immunizations of dm2 mice with the pCI/S-A DNA vaccine, nor after repeated in vitro restimulation of CD8 T cells from immunized mice with one to three antigenic S201–209 peptides (data not shown). Stimulating spleen cells from pCI/S-D-primed mice with titrated amounts of each variant of the antigenic S201–209 peptide revealed that the variant D peptide restimulated CD8 T cells more efficiently than variant A peptide (Fig. 2B). This was confirmed by restimulating pCI/S-D-primed, S201–209-specific short-term CD8 T cell lines with P815 cells pulsed with titrated doses of the Dd-binding S201–209 peptide variants and measuring IFN-γ release (Fig. 2B). Dd-restricted S201–209-specific CD8 T cells are thus primed by variant D but not variant A, and CD8 T cells primed by variant D showed relatively low cross-reactive recognition of the variant A epitope. To determine whether the inability to prime variant A-specific CD8 T cells could be due to an Ag-processing and/or presentation defect, P815 transfectants were generated that express comparable levels of the A or D variant of HBsAg (Fig. 3A). In specific cytolytic or cytokine release assays, pCI/S-D-primed CD8 T cells responded to transfectants expressing variant D but not transfectants expressing variant A. These results strongly suggest that the loss of the consensus P9 anchor residue reduces processing/presentation efficacy of the A variant peptide.

**FIGURE 1.** Dd-restricted CD8⁺ T cell responses to natural variants D and C of the Dd-restricted S201-209 epitope of HBsAg by DNA vaccines. A, Priming CD8 T cell responses to natural variants D and C of the Dd-restricted S201-209 epitope of HBsAg by DNA vaccines. dm2 mice were vaccinated i.m. with 100 µg/mouse pCI, pCI/S-D, or pCI/S-C plasmids. Spleen cells obtained 12-day after vaccination were restimulated for 5 h (in the presence of brefeldin A) with excess (2500 nM) Dd-binding S201–209 peptide of the HBsAg variant D (WGPSLYSIL) or C (WGPSLYNIL). As a control, spleen cells were stimulated with an irrelevant Dd-restricted peptide from HIV gp120 (RGPGRAFVTI). IFN-γ⁺CD8⁺ T cells were detected by flow cytometry. Mean numbers of splenic IFN-γ⁺CD8⁺ T cells/10⁵ CD8⁺ T cells ± SD of four mice per group are shown. B, Fine specificity of CD8 T cells reactive to natural variants of the Dd-restricted S201-209 epitope of HBsAg. Furthermore, pCI/S-D or pCI (expression vector without insert)-primed T cells were stimulated ex vivo with titrated amounts of the S201–209 peptides from variant D (WGPSLYSIL) and IFN-γ⁺CD8⁺ T cells (left panel) or TNF-α⁺CD8⁺ T cells (right panel) were detected by flow cytometry as described above. Furthermore, pCI/S-D or pCI (expression vector without insert)-primed T cells were stimulated ex vivo with titrated amounts of the S201–209 peptides from variant D or C, and IFN-γ⁺CD8⁺ T cells were determined.
CD8 T cells cross-reactive to the two natural variants C and D of the Dd-restricted S201–209 determinant show preferential reactivity to the priming epitope

CD8 T cells primed to the variant C (WGPSLYNIL) recognized the variant D epitope naturally processed by transfected P815/S-D cells (Fig. 3B). Thus, a natural epitope variant of HBsAg with a single residue exchange (WGPSLYNIL vs WGPSLYSIL) primes cross-reactive CD8 T cell immunity to a naturally processed variant of the epitope. As expected, transfectants expressing variant A did not present this epitope to immune CD8 T cells (Fig. 3B).

Recognition of the tested variant S201–209 peptides is Dd restricted

To confirm that all variant S201–209 peptides tested were presented in the context of the Dd class I molecule, CIR transfectants were pulsed with peptides and used to restimulate immune spleen cells primed by pCI/S-C. The variant S201–209 peptides were presented by peptide-pulsed Dd-, but not Kd-expressing CIR cells (Fig. 4). Similar data were obtained when immune spleen cells primed by pCI/S-D were restimulated ex vivo by peptide-pulsed CIR transfectants (data not shown). A change in the presenting restriction element thus does not underlie variant-specific recognition of the S201–209 epitope.

CD8 T cell tolerance to the variant D of the S201–209 epitope is overcome by priming immunity to the variant C epitope

pAlb-HBs mice express the HBsAg D variant from a transgene in the liver (18, 24, 27). Due to the transgenic nature of the HBsAg, pAlb-HBs mice are immunologically tolerant to the D variant.
We examined whether CD8 T cells specific for variant C can be primed in these transgenic mice and, if so, whether the primed CD8 T cells are able to cross-react with the variant D epitope of HBsAg constitutively produced by these animals. Transgenic F1 mice were generated by crossing pAlb-HBs B6 mice with dm2 mice and selecting for HBsAg+/H11001 progeny. Transgenic (HBsAg+/H11001) and nontransgenic (HBsAg+/H11002) F1 mice were repeatedly vaccinated with the pCI/S-C or pCI/S-D DNA vaccines. As expected, the pCI/S-D vaccine primed a CD8 T cell response to the variant D S201–209 epitope in nontransgenic but not transgenic F1 mice (Fig. 5, group 2) (28). In contrast, vaccination of transgenic F1 mice with the pCI/S-C vaccine induced a CD8 T cell response to variant C of the S201–209 epitope (Fig. 5B, group 4). Although these CD8 T cells preferentially recognized the variant C epitope, they showed cross-reactivity to the variant D epitope (Fig. 5B, group 4). This response was lower than that elicited by the pCI/S-C vaccine in nontransgenic F1 mice (Fig. 5A, group 4). Thus, a natural epitope variant of HBsAg with a single residue exchange (WGPSLYNIL vs WGPSLYSIL) can prime cross-reactive CD8 T cell immunity in a mouse tolerant to one variant of the epitope.

We primed CD8 T cell responses to HBsAg variants in HLA A*0201-transgenic mice to test whether residue exchanges within A2-restricted epitopes of natural HBsAg variants change its immunogenicity and/or cross-reactivity. We selected for this study the A and D variants of the HLA A2-restricted S185–194 (29) and S208–216 (30) epitopes because they contain residue exchanges within the antigenic determinant. Similar to the A variant Dd-restricted epitope, the A variant S185–194 epitope has a modified C-terminal anchor residue (exchange of a valine V in variant D/C into an alanine A in variant A) (Table II). A2-tg mice were vaccinated with the pCI/S-A or the pCI/S-D vaccine. The pCI/S-D vaccine primed CD8 T cells to the S185–194 epitope that were restimulated ex vivo efficiently by the variant D GLSPTVWLSV peptide but inefficiently by variant A GLSPTVWLSA peptide (Fig. 6). No S185–194-specific CD8 T cell response was primed by the pCI/S-A vaccine (Fig. 6). Thus, similar to priming the D4-restricted S201–209-specific response to HBsAg in dm2 mice, a residue exchange in an anchor position strikingly reduced the immunogenicity of this HLA A2-restricted epitope in vivo and its antigenicity in vitro (cf Figs. 2 and 6). We used the 9-mer S208–216 epitope ILSPFLPLL (modified from a published sequence (30) by omitting the N-terminal S) since this optimally restimulates CD8 T cells (Fig. 7A). The A

S201–209 epitope. We examined whether CD8 T cells specific for variant C can be primed in these transgenic mice and, if so, whether the primed CD8 T cells are able to cross-react with the variant D epitope of HBsAg constitutively produced by these animals. Transgenic F1 mice were generated by crossing pAlb-HBs B6 mice with dm2 mice and selecting for HBsAg+ progeny. Transgenic (HBsAg+) and nontransgenic (HBsAg-) F1 mice were repeatedly vaccinated with the pCI/S-C or pCI/S-D DNA vaccines encoding the C or D variants of HBsAg. As expected, the pCI/S-D vaccine primed a CD8 T cell response to the variant D S201–209 epitope in nontransgenic but not transgenic F1 mice (Fig. 5, group 2) (28). In contrast, vaccination of transgenic F1 mice with the pCI/S-C vaccine induced a CD8 T cell response to variant C of the S201–209 epitope (Fig. 5B, group 4). Although these CD8 T cells preferentially recognized the variant C epitope, they showed cross-reactivity to the variant D epitope (Fig. 5B, group 4). This response was lower than that elicited by the pCI/S-C vaccine in nontransgenic F1 mice (Fig. 5A, group 4). Thus, a natural epitope variant of HBsAg with a single residue exchange (WGPSLYNIL vs WGPSLYSIL) can prime cross-reactive CD8 T cell immunity in a mouse tolerant to one variant of the epitope.

Priming CD8 T cell responses to HBsAg variants in HLA A*0201-transgenic mice

We primed CD8 T cell responses to HBsAg variants in HLA A*0201-transgenic (A2-tg) mice to test whether residue exchanges within A2-restricted epitopes of natural HBsAg variants change its immunogenicity and/or cross-reactivity. We selected for this study the A and D variants of the HLA A2-restricted S185–194 (29) and S208–216 (30) epitopes because they contain residue exchanges within the antigenic determinant. Similar to the A variant Dd-restricted epitope, the A variant S185–194 epitope has a modified C-terminal anchor residue (exchange of a valine V in variant D/C into an alanine A in variant A) (Table II). A2-tg mice were vaccinated with the pCI/S-A or the pCI/S-D vaccine. The pCI/S-D vaccine primed CD8 T cells to the S185–194 epitope that were restimulated ex vivo efficiently by the variant D GLSPTVWLSV peptide but inefficiently by variant A GLSPTVWLSA peptide (Fig. 6). No S185–194-specific CD8 T cell response was primed by the pCI/S-A vaccine (Fig. 6). Thus, similar to priming the D4-restricted S201–209-specific response to HBsAg in dm2 mice, a residue exchange in an anchor position strikingly reduced the immunogenicity of this HLA A2-restricted epitope in vivo and its antigenicity in vitro (cf Figs. 2 and 6). We used the 9-mer S208–216 epitope ILSPFLPLL (modified from a published sequence (30) by omitting the N-terminal S) since this optimally restimulates CD8 T cells (Fig. 7A). The A
variant S\textsubscript{208–216} epitope has exchanges in the N-terminal P2 (exchange of a leucine L in variant D/C into a valine V in variant A) and the auxiliary P6 anchor residue (exchange of a leucine L in variant D/C into an isoleucine I in variant A) (Table II). Immunization of A2-tg mice with the pCI/S-A or pCI/S-D vaccines primed A2-restricted S\textsubscript{208–216}-specific CD8 T cells. Although, the specificity analysis revealed that the CD8 T cells preferentially recognized the S\textsubscript{208–216}peptide variant to which they were primed (variant D/C IL\textsubscript{2}SPFLPLL and variant A IVSPFIPLL), variant D/C-primed CD8 cells recognized the variant A peptide and variant A-primed CD8 cells recognized variant D/C peptide (Fig. 7B). Thus, similar to priming D\textsuperscript{d}-restricted S\textsubscript{201–209}-specific responses to variant D or C in dm\textsubscript{2} mice, the A2-restricted CD8 T cell response to S\textsubscript{208–216} elicited by variant A or variant D displayed cross-reactivity but also preferential recognition of the immunizing epitope variant (cf Figs. 1 and 7B).

Cross-reactive HBSAg-specific CD8 T cells induced by natural HBV infection

Having demonstrated that natural variants of the HLA-A2-restricted S\textsubscript{208–216} epitope can induce cross-reactive CD8 T cells in A2-transgenic mice, we investigated whether similar cross-reactive CD8 T cells are induced in natural HBV infection. We initially tested whether the two S\textsubscript{208–216} variant peptides (variant D/C IL\textsubscript{2}SPFLPLL and variant A IVSPFIPLL) can expand S\textsubscript{208–216}-specific CD8 T cells from HBV-infected patients. The incidence of the S\textsubscript{208–216}-specific CD8 response in A2\textsubscript{+} patients who resolved HBV infection was low (2 of 18 subjects tested positive). However, in both S\textsubscript{208–216}-responsive subjects, restimulation with the variant A and the variant D S\textsubscript{208–216} peptide specifically activated CD8 T cells (Fig. 8A), indicating that S\textsubscript{208–216} (cross-)reactive CD8 cells can be induced by natural HBV infection. Interestingly, the S\textsubscript{208–216}-A variant elicited stronger CD8 T cell expansion than the S\textsubscript{208–216}-D variant peptide in both patients. Because the S\textsubscript{208–216}-A variant peptide is derived from the HBV genotype A, the more common genotype present in our geographical area (North Europe) (9, 10), it is likely that the immunogenic difference of the two variant peptides reflects the initial infection with genotype A.

To further confirm the cross-reactive pattern of S\textsubscript{208–216}-specific CD8 T cells induced by natural HBV infection, we selected and expanded in vitro S\textsubscript{208–216}variant A-specific CD8 T cells from an HLA-A2\textsubscript{+} patient with resolved HBV infection. The ability of S\textsubscript{208–216} variant A-specific CD8 clones to recognize the S\textsubscript{208–216} variant D peptide was tested in vitro by a novel technique that...
FIGURE 7. Priming CD8 T cell responses to natural variants D and A of the HLA-A2-restricted S208–216 epitope of HBsAg by DNA vaccines. A, Characterization of the env 10 epitope. A2-tg mice were vaccinated i.m. with 100 μg/mouse pCDS/A plasmid. Spleen cells obtained 12 days after vaccination were restimulated for 5 h (in the presence of brefeldin A) with indicated amounts of the S207–216 peptide of the HBsAg-A variant (SVSPFLPLL) previously described as env 10 (29) or with a shortened S208–216 peptide (IVSPFLPLL). IFN-γ+/CD8+ T cells were detected by flow cytometry. Mean numbers of splenic IFN-γ+CD8+ T cells/10^6 CD8+ T cells ± SD of three mice per group are shown. B, Induction of S208–216 specific CD8 T cell responses with distinct natural epitope specificities. A2-tg mice were vaccinated i.m. with 100 μg/mouse pCD5-D or pCD5-A plasmids. Spleen cells obtained 12 days after vaccination were restimulated for 5 h (in the presence of brefeldin A) with indicated amounts of the A (IVSPFLPLL) or D (ILSPFLPLL) variant of the S208–216 peptide. IFN-γ+CD8+ T cells were detected by flow cytometry. Mean numbers of splenic IFN-γ+CD8+ T cells/10^6 CD8+ T cells ± SD of four mice per group are shown.

Discussion

Natural, single residue exchanges in the sequence of antigenic peptides from HBsAg that bind murine Dd or human HLA-A*0201 class I molecules can change their immunogenicity for CD8 T cells following activation with cognate peptide, concordant with production of intracellular IFN-γ. The loss of consensus anchor residues in Dd- or HLA A2-binding HBsAg epitopes abrogated their immunogenicity. In contrast, conservative exchanges in the sequence of the antigenic peptide from the three tested variants efficiently primed apparently distinct but cross-reactive CD8 T cell populations. Preferential reactivity against the priming variant of the antigenic peptide and successful CD8 T cell priming to a variant in a mouse tolerant to the alternative variant of the same peptide provides evidence that distinct T cell populations were specifically activated by each variant epitope. Similar data were found in HBV-infected patients.

Generation of variant epitopes of Ags during a virus infection is widespread although the extent of this variability differs greatly

FIGURE 8. Cross-reactive S208–216 specific CD8+ T cells induced by natural HBV infection. A, In vitro activation of S208–216 variant D- and A-specific CD8+ cells. PBMC of two HLA-A2+ patients who resolved HBV infection (anti-HBs+; anti-HBc–) were stimulated with 1 μM of the indicated peptides. After 10 days of in vitro expansion, the PBMC were restimulated with the initial stimulatory peptides or with irrelevant peptide (HCV NS3 1073-81). Frequency of IFN-γ-producing CD8+ cells was analyzed by flow cytometry. Bars indicate the frequency of IFN-γ+specific IFN-γ-producing cells subtrahed by the background obtained in cells stimulated with the irrelevant peptide (<0.2%). B, Recognition of the S208–216 variant D peptide by S208–216 variant A-specific CD8+ T cell clones. HLA-A2+ EBV-B cells were pulsed for 1 h with the indicated peptide concentrations. After extensive washing, peptide-pulsed target cells were coincubated with S208–216 variant A-specific CD8+ cells at a 1:1 target:effector ratio. After 4 h, the CD107 expression of CD8+ T cells (left panel) or IFN-γ expression of CD8+ T cells (right panel) was analyzed by flow cytometry. Parameters for CD107 expression analysis were calculated on CD8+ cells incubated with peptide-unpulsed EBV-B cells.
among viruses. An immune response against a variant epitope may be generated either by eliciting a polyclonal primary response comprising (possibly infrequent and subdominant) cross-reactive specificities recognizing the variant determinant, or by de novo priming of a variant-specific response when the variant arises. An example for the former has been published in the influenza system (31). The polyclonal CD8 T cell population specific for an influenza A nucleoprotein epitope contains infrequent cross-reactive T cells that expand from the original memory population upon challenge with a variant virus to produce T cells productively recognizing both the parental and the mutant epitope. Polyclonal memory T cell populations can thus provide protection against a range of antigenic variants. This may not hold true for chronic virus infection where the relevant cross-reactive T cells are eliminated. Under these conditions, the alternative approach of eliciting a new and strong, variant-specific response with cross-reactivity toward the infecting variant may be more promising. As demonstrated in the present study, this approach can even break tolerance, supporting a potential therapeutic value of this strategy.

MHC molecules show a broad specificity for peptide binding. Consensus anchor residues that mediate peptide binding to MHC molecules are required to make peptides antigenic. This is confirmed in the present study for Dd and HLA A2-binding HBsAg peptide; eliminating one of their consensus anchors eliminates their antigenicity. The fine specificity of Ag recognition resides in TCR contact residues that project from the peptide-binding cleft of the MHC molecules. Restricted TCR recognition of MHC molecule plus peptide displays exquisite specificity but also degeneracy. This has become apparent especially by studies on T cell clones that can show unexpected degeneracy as well as fine specificity. For example, the C2 TCR clone recognizes at least 12 antigenic peptides in the context of three restricting MHC proteins (K\textsuperscript{b}, L\textsuperscript{d}, and H-2\textsuperscript{d}), but also displays exquisite specificity by discriminating between very similar epitopes (32). As described by the “altered peptide ligand” concept, stimulation of T cells by their cognate peptide into which single, conservative residue exchanges have been introduced often results in only partial activation, changes in phenotype and/or anergy triggered by qualitatively different signal transduction events (33). Variant peptides can, therefore, trigger responses in cross-reactive T cells that are not necessarily protective. It seems furthermore important to consider how many different T cell clones are specifically activated in a T cell response to an epitope. This would give information on the repertoire of clonotypes from which cross-reactive T cells to particular variants of an epitope can be recruited. Published data (34) (involving single-cell PCR analyses of the TCR of specific cells) suggest that only few clones are present at the peak of the primary response to an individual epitope, and these clones also predominate in the subsequent memory response. But this may vary substantially between individual Ags. In vivo, rather few clones with a specificity of unknown degeneracy are thus expected to be primed by an epitope, some of which have the chance to become eliminated because they only partially activate a T cell.

The emergence of residue exchanges within an epitope would be expected to have many alternative outcomes: 1) primed T cell clones cross-react to the new variant epitope and are fully activated; 2) primed clones cross-react to the variant epitope but are only partially activated or eliminated; 3) previously, only partially activated clones become fully activated; 4) the variant is not immunogenic; or 5) new T cell clones are primed. Many reports demonstrated that single residue exchanges within an antigenic peptide can make it more immunogenic (and therapeutically more efficient), as shown for the HLA-A2-restricted p53\textsubscript{264–272} epitope of a tumor-associated Ag (35). We published similar findings using a natural variant of a K\textsuperscript{b}-restricted HBsAg epitope: a variant with a higher affinity for K\textsuperscript{b} could overcome specific tolerance established by a variant of the peptide with lower affinity for K\textsuperscript{b} (13, 14). The study described in this article shows that variant-induced T cell populations are restimulated more efficiently by the variant that primed them than by alternative, natural variants. Importantly, the new clonotypes establish cross-reactive CD8 T cell immunity to alternative variants even in a mouse tolerant to these alternative variants. It is the specific but degenerate recognition of T cells primed to variants of the same epitope that makes this approach an interesting candidate for therapeutic vaccination.

Can variation of viral Ags through limited single residue exchanges produce changes in the immunogenicity of the Ag by other means? We reported in the HBsAg system three possible, additional ways by which variation of the Ag can enhance the immunogenicity of the surface Ag and override the tolerance that prevails in chronic infection. The first (described above) is the generation of variants with higher affinity for their restricting element. The second involves attenuation of immunodominant (suppressive) epitopes (22–24) that may reveal a large panel of subdominant (or cryptic) epitopes. The third involves residue exchanges in epitope-flanking sequences that are permissive or inhibit processing of the antigenic peptide (13, 14). Thus, different options are available to rationally design variant Ag constructs for the specific therapy of chronic virus infection.

Single, conservative residue exchanges presumably within TCR contact regions of the epitope can thus prime distinct but cross-reactive CD8 T cell immunity to HBV in mice, and these cross-reactive T cells can escape tolerance induction in transgenic mice. It will be critical to demonstrate in transgenic mice that cross-reactive, variant-specific CD8 T cells exert liver-specific, antiviral effector functions. If so, the data would support the notion that HBsAg variants are attractive candidates for therapeutic immunization. It was equally important to show in this study that HBV envelope-specific CD8 T cells able to recognize peptides with conservative variants are primed during natural infection. This demonstrates that CTL cross-reactivity between envelope epitopes is not restricted to mice but is naturally present in patients who recovered from HBV infection. Whether cross-reactive, HBV-specific CD8 T cells play a role in the final control of infection is not known, but this can be potentially tested in the spontaneous remission of chronic HBV infection in patients. HBV variants have been shown to be present during acute flare-ups of HBV chronic infection, leading to hepatitis B Ag seroconversion (36). The possibility exists that HBV genetic variability, which potentially increases at the time of hepatitis B e Ag seroconversion, produces new immunogenic epitopes able to break HBV-specific tolerance in chronic hepatitis B patients. Such evidence would support our assumption on the usefulness of variant epitopes in the specific immunotherapy of the chronic infection.

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Disclosures

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