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Multiepitopic HLA-A*0201-Restricted Immune Response Against Hepatitis B Surface Antigen After DNA-Based Immunization

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CTL together with anti-envelope Abs represent major effectors for viral clearance during hepatitis B virus (HBV) infection. The induction of strong cytotoxic and Ab responses against the envelope proteins after DNA-based immunization has been proposed as a promising therapeutic approach to mediate viral clearance in chronically infected patients. Here, we studied the CTL responses against previously described hepatitis B surface Ag (HBsAg)-HLA-A*0201-restricted epitopes after DNA-based immunization in HLA-A*0201 transgenic mice. The animal model used was Human Human D\(^{b}\) (HHD) mice, which are deficient for mouse MHC class I molecules (\(\beta_2\)-microglobulin \(^{-/-}\)\(D^{b/-/-}\)) and transgenic for a chimeric HLA-A*0201/D\(^{b}\) molecule covalently bound to the human \(\beta_2\)-microglobulin (HHD\(^{+/-}\)). Immunization of these mice with a DNA vector encoding the small and the middle HBV envelope proteins carrying HBsAg induced CTL responses against several epitopes in each animal. This study performed on a large number of animals described dominant epitopes with specific CTL induced in all animals and others with a weaker frequency of recognition. These results confirmed the relevance of the HHD transgenic mouse model in the assessment of vaccine constructs for human use. Moreover, genetic immunization of HLA-A2 transgenic mice generates IFN-\(\gamma\)-secreting CD8\(^{+}\) T lymphocytes specific for endogenously processed peptides and with recognition specificities similar to those described during self-limited infection in humans. This suggests that responses induced by DNA immunization could have the same immune potential as those developing during natural HBV infection in human patients. The Journal of Immunology, 2000, 165: 4748–4755.

Hepatitis B virus (HBV) infection with 350 million carriers in the world represents an important health problem (1). This infection leads in some carriers to cirrhosis and ultimately to hepatocellular carcinoma (2). Most patients infected as adults develop a self-limited acute hepatitis. In acutely infected patients the immune response includes high Ab titers against the core and the envelope proteins as well as vigorous, polyclonal, and multispecific cellular responses (3). On the other hand, in patients who do not clear the viral infection or who were infected early in life and become chronic carriers, the cellular response is undetectable or weak and is restricted to few antigenic determinants (4). Several observations suggest that cytotoxic T cells are required for the viral clearance, although lysis of infected hepatocytes is unlikely to be the only mechanism through which CD8\(^{+}\) T cells exert their anti-viral effect. Indeed, in transgenic mouse models for HBV gene expression and/or replication, CTL mediate not only the down-regulation of viral gene expression (5, 6) but also the control of viral replication (7). This phenomenon is probably due to the anti-viral effects of Th1 cytokines (i.e., IFN-\(\gamma\), IFN-\(\alpha\), IFN-\(\beta\), and TNF-\(\alpha\)) secreted by CD8\(^{+}\) T cells after antigenic stimulation or following a concomitant viral infection (8).

At present, there is no efficient cure for HBV chronic infections. IFN-\(\alpha\) treatment is efficient in only 40% of patients, and treatment with nucleoside analogues leads to the development of drug resistance in some patients. Specific immunotherapies such as injection of envelope-based-recombinant vaccine (9) or core-specific lipopeptides (10, 11) have been designed to induce or to boost cellular responses in chronically infected patients.

DNA-based immunization represents a promising approach to induce specific cytotoxic responses. It is achieved by intradermal or i.m. injection of DNA plasmids encoding one or several Ags. The in vivo production of the Ag leads to a complete immune response, with production of Abs and the initiation of long-lasting CD4\(^{+}\) and CD8\(^{+}\)-specific T cell responses (12). We have developed DNA immunization against HBV based on injection of vectors encoding hepatitis B surface Ag (HBsAg) (13, 14). To date, several constructs have been tested in different animal models (mice and nonhuman primates) in which Abs (15, 16), CD4\(^{+}\) T cell proliferative responses (17), and CD8\(^{+}\) CTL were induced (18–20). Results obtained in HBsAg-transgenic mice, an animal model for HBV chronic carriers, suggested that DNA-based immunization against HBsAg could induce or increase the immune response in chronically infected patients (6, 17). These results have been further confirmed in duck and woodchuck as animal models for hepadnavirus infection (21, 22).

Before applying genetic immunization approaches to humans, we sought to verify that injection of a plasmid encoding an Ag allows the generation of cytotoxic T cells of specificities comparable to those observed in infected patients. To address this question, we studied the specificity of cytotoxic responses induced in HLA-A*0201 transgenic mice (23) by DNA immunization with...
regard to that observed during acute infection in humans (3, 24). In this study we have analyzed the cytotoxic responses against previously defined HLA-A*0201-restricted epitopes of HBsAg after a single injection of a plasmid encoding the small and the middle HBV envelope proteins, both carrying the HBsAg. This was performed in human dendritic cells (HHD) mice derived from a strain deficient for the β2-microglobulin (β2-m) gene and the mouse MHC class I-2D* molecules and transgenic for a chimeric HLA-A*0201/D* molecule. In these animals the chimeric molecule is the only class I molecule serologically detected on cell surfaces (23). After infection or immunization, these mice have been shown to be a relevant animal model for HLA-A*0201-restricted epitope presentation (25, 26).

Our results demonstrate that the CD8+ T cell response induced by injection of plasmids encoding the HBV envelope proteins in HHD mice is multiepitopic and that its specificity mimics the responses of HBV-infected patients.

Materials and Methods

HHD transgenic mice

Female HHD+/+ β2-m−/− D*−/− mice used for immunization were 6–8 wk old. The β2-m−/− D*−/− strain was made transgenic for a chimeric HLA-A*0201 monochain, the HHD molecule (23) in which the human β2-m molecule is covalently linked to the NH2 terminus of a hybrid MHC class I heavy chain. The hybrid heavy chain is composed of the α1 and α2 domains of the human HLA-A0201 MHC class I molecule and the α3, transmembrane, and cytoplasmic domains of the mouse H-2D* MHC class I molecule. The expression of this monomeric MHC class I molecule on the cell surfaces in β2-m- and H-2D-deficient mice restores a partial CD8+ population in the spleen that represents 5% of total splenocytes.

Peptides and immunization

Peptides synthesized by Neosystem (Strasbourg, France) were dissolved in PBS-10% DMSO at a concentration of 2 mg/ml. Groups of five to nine mice were immunized once with 50 μg of T helper peptide (hepatitis B nucleocapside, HBc128–140 TPPATRPPNAPIL) emulsified in IFA (Difco, Detroit, MI). The emulsion (100 μl) was injected s.c. at the base of the tail (27). For loading LPS blasts and target cells, peptides were dissolved in RPMI-20% FCS. For each peptide, the concentration needed to reach 20% maximal binding (27) was determined from wells containing either medium alone or lysis buffer (30). Each muscle was injected with 50 μg of DNA. All i.m. injections were conducted under anesthesia (sodium pentobarbital, 75 mg/kg i.p.). All experiments involving mice were conducted in accordance with institutional guidelines.

Cell lines

All cell lines were cultured in RPMI 1640 medium (Life Technologies, Cergy-Pontoise, France) supplemented with 10% FCS, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 2 mM l-glutamine (CM). HeLa and RMA-S cells were transfected with the HHD encoding construct as previously described (23), and PB15 cells were transfected with the native HLA-A*0201 and human β2-m genes. Cell surface expression of the HHD monochain or the HLA-A*0201 molecule was verified by indirect immunofluorescence using mAb BB7.2 and anti-mouse IgG (Diagnostic Pasteur) and was monitored by FACS analysis. The LPS blast cells were obtained after red cell depletion by culturing splenocytes of nonimmunized syngeneic mice in CM supplemented with 5 × 10–3 M 2-ME; 1 mM sodium pyruvate, LPS from Salmonella typhosis (25 μg/ml Sigma, St. Louis, MO), and dextran sulfate (7 μg/ml Pharmacia, Uppsala, Sweden). After 3 days of culture, splenocytes were enriched with mature B cells. The cells were washed with serum-free medium and incubated for 3 h with the different HLA-A*0201 peptides (10 μg/ml) at room temperature. The LPS blasts were irradiated (40,000 rad) before coculture with splenocytes from immunized mice.

Measurement of CTL activity

Spleens were removed 7 or 8 days after peptide immunization or 3 wk after DNA immunization. RBC-depleted splenocytes (0.83% NH4Cl, 5 min, on ice) were cultured (5 × 105 cells in 24-well plates) in α-MEM (Life Technologies) supplemented with 10 mM HEPES, nonessential amino acids, 1 mM sodium pyruvate, 5 × 10–3 M 2-ME, antibiotics, and 10% FCS (Myochine, Life Technologies). These cells were stimulated for 6–7 days with peptide-loaded LPS-blast cells as APCs at an effector:presenting cell ratio of 2:1 to 1:1. The specific cytolytic activity of effector cells was tested in a short term 51Cr release assay against either RMA-S HHD or HeLa HHD target cells pulsed with 10 μg/ml of the various HLA-A*0201 peptides derived from the HBV small envelope protein or target cells pulsed with the well-described HLA-A*0201 HBV core epitope (HBC127–135) as a negative control. After a 4-h incubation at 37°C, supernatant was collected and counted on a beta counter. Supernatant and maximum releases were determined from wells containing either medium alone or lysis buffer (5% Triton and 1% SDS). Specific lysis was calculated in triplicate as [(experimental – spontaneous release)/(maximum – spontaneous release)] × 100. For some experiments secondary in vitro restimulations were performed for an additional week as described above, except that 5% T cell growth factor was added.

IFN-γ enzyme-linked immunospot (ELISPOT) assay

IFN-γ-releasing cells were quantified after peptide- or vaccinia virus-injected cell stimulation by cytokine-specific ELISPOT assay. Ninety-six-well nitrocellulose-backed plates (Multiscreen, Millipore, Molsheim, France) were coated with 50 μl of mouse IFN-γ mAb (5 μg/ml; Pharmingen, San Diego, CA) overnight at 4°C, then washed three times with serum-free medium and blocked for 2 h using CM. After red cell lysis, freshly isolated undepleted or CD8−-depleted splenocytes (1 × 105/well) were incubated for 4 h in complete α-MEM medium (see CTL activity) at 37°C in 5% CO2 using different antigenic stimulations. Cells were incubated with either HLA-A*0201-restricted peptide (1 μg/ml; see Table I) or a mouse MHC class II-restricted peptide (HBC127–135) derived from the HBV envelope middle protein (3 μg/ml) (17) or were infected with a recombinant vaccinia virus encoding the three HBV envelope proteins (rVSV S1 S2 S) (29) at a multiplicity of infection (MOI) of 1/100 (30). Quadruplicate determinations were made for Ag-stimulated splenocytes, and triplicate determinations were made for unstimulated splenocytes or for splenocytes infected with wild-type vaccinia virus to determine background. Positive controls were made by adding Con A (5 μg/ml) to the stimulation wells. After incubation, cells were lysed with 4% SDS for 15 min at room temperature. The wells were washed three times each with PBS-Tween or PBS before adding secondary biotin-conjugated Ab (biotinylated anti-murine IFN-γ, Pharmingen) for 90 min at room temperature. The wells were washed as described above before incubation with alkaline phosphatase-conjugated streptavidin (Roche, Mannheim, Germany) at a 1/1000 dilution in PBS for 1.5 h. After rewashing as before, spots were developed by adding peroxidase substrates, 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Promega, Madison, WI). After the appearance of spots (30 min to 1 h) the reaction was stopped with tap water and air-dried. Spots...
were counted in a double-blinded fashion as single spot-forming cells (SFC) under a stereo binocular. The number of Ag-specific IFN-γ-secretory CD8+ T cells was determined by subtracting the number of background spots from the number of spots found for splenocytes stimulated with peptide or infected with rVV-S1.S2.S. The percentage of CD8+ T cells was determined by FACS analysis of fresh splenocytes using an anti-mouse CD8+ FITC Ab (PharMingen). Depletion of CD8+ T cells from mouse splenocytes was achieved by magnetic cell sorting (MACS; Miltenyi Biotech, Paris, France) as previously described (17). The percentage of CD8+ T cells in the depleted fraction was <0.4%. Results

Relative affinity of HBV-envelope peptides for the HLA-A*0201 molecule

We have analyzed the capacity of eight HBV-envelope HLA-A*0201 peptides described as epitopes in humans to bind and stabilize the HLA-A*0201 molecule on the T2 cell surface. T2 cells are HLA-A*0201 positive and TAP-negative human cells expressing unstable HLA-A*0201 molecules at 37°C. Providing exogenous peptides susceptible to bind HLA-A*0201 molecules results in their stabilization. Cells were incubated overnight with peptide at various concentrations, and the number of stabilized HLA-A*0201 molecules on cell surface was quantified by FACS analysis. This test allowed us to calculate the relative affinity for the HLA-A*0201 molecule of each tested peptide compared with a reference peptide (HBc18–27) displaying a high affinity (27). The relative affinity for all peptides given in Table I was the mean of two individual experiments. These results allowed us to distinguish peptides with high (HBs260–269, HBs335–343, HBs348–357), intermediate (HBs177–185, HBs204–212, HBs370–379), and very low (HBs177–185 and HBs251–259) affinities.

T cells from HHD mice recognize the HBV envelope HLA-A*0201-restricted epitopes

To evaluate whether the T cell repertoire of the HHD transgenic mice is wide enough to recognize all HLA-A*0201-restricted epitopes described previously in HBV-infected humans, mice were immunized separately with the eight peptides corresponding to HLA-A*0201 epitopes of the small HBV envelope protein (see Table I). To optimize the cytotoxic responses, we coinjected the HLA-A*0201-restricted peptides with a T helper peptide in IFA. After one s.c. peptide injection and one in vitro peptide restimulation, we detected specific cytotoxicity against all peptides tested (Fig. 1), indicating that the eight HBV epitopes were efficiently presented by the chimeric HHD molecule and that the HHD/envelope complex was well recognized by TCR. Most peptides (HBs177–185, HBs183–191, HBs204–212, HBs260–269, HBs348–357, and HBs370–379) induced a strong cytotoxic response in almost all immunized mice, whereas two peptides (HBs251–259 and HBs335–343) induced a weaker response in some injected mice. However, the weak response induced by injection of the peptide HBs251–259 could be related to its very low affinity for HLA-A*0201 molecule (Table I), which is probably linked to peptide dimerization by cysteine (31). It is surprising that the peptide HBs335–343, which belongs to the high affinity group (Table I), did not induce a strong CTL activity. Thus, no absolute correlation was found between the HLA-A*0201 binding capacity and the frequency or intensity of the CTL response. Nevertheless, cytotoxic activity could be elicited against each tested peptide, indicating that despite the low number of CD8+ T cells, the HLA-A*0201-educated repertoire of HHD mice is wide enough to allow recognition of all envelope-derived epitopes that have been described in HBV-infected human.

Table I. HLA-A*0201 peptide-binding and stabilizing capacities of HBV envelope-derived epitopes

<table>
<thead>
<tr>
<th>Position1</th>
<th>Amino Acid Sequence2</th>
<th>RA3</th>
<th>Ref.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>177–185</td>
<td>VLGQAGFILL</td>
<td>441.3</td>
<td>3</td>
</tr>
<tr>
<td>183–191</td>
<td>FLLTRILT</td>
<td>14.28 ± 2.98</td>
<td>3</td>
</tr>
<tr>
<td>204–212</td>
<td>FLGGTPVCVL</td>
<td>4.07</td>
<td>3</td>
</tr>
<tr>
<td>251–259</td>
<td>LLCLIFILLV</td>
<td>&gt;450</td>
<td>24</td>
</tr>
<tr>
<td>260–269</td>
<td>LLDYQGMLP</td>
<td>1.68 ± 0.02</td>
<td>24</td>
</tr>
<tr>
<td>335–343</td>
<td>WLSLIVPVF</td>
<td>1.41 ± 0.11</td>
<td>24</td>
</tr>
<tr>
<td>348–357</td>
<td>GLSPTYWLS</td>
<td>1.12 ± 0.12</td>
<td>24</td>
</tr>
<tr>
<td>370–379</td>
<td>SIVSFSIPPLL</td>
<td>5.27 ± 0.7</td>
<td>3</td>
</tr>
</tbody>
</table>

1 Position of peptides on the small HBV envelope protein are indicated starting from the first methionine of the PreS1 region in the HBV sequence. All peptides are located in the S gene encoding the HBsAg.
2 Amino acid sequence of the peptides. Amino acids involved in the binding to the HLA-A*0201 molecule are in bold.
3 RA is the ratio of the concentration of tested vs reference peptides needed to reach 20% of the maximal amount of stabilized molecules as defined with high concentrations of reference peptide (see Materials and Methods).
4 Reference corresponding to the first description of a cytotoxic response specific for the epitope in HBV-infected patients.

Figure 1. CTL response against HLA-A*0201-restricted HBV envelope epitopes after single peptide immunization. The cytotoxic activity of effectector cells was measured against RMA-S HHD cells pulsed with HLA-A2 peptides. Specific lysis was calculated by subtracting the nonspecific lysis on HBCAg peptide-loaded targets (control) from the specific lysis obtained on HBsAg peptide-loaded targets. Cytolytic responses were considered positive (filled columns) when the specific lysis was ≥10% at an E:T cell ratio of 100:1. Panels represent specific lysis for an E:T cell ratio of 100:1 in five to nine independently tested mice immunized with the same HBsAg peptide. The positions of the peptides on the HBV envelope protein are indicated under each panel. The numbers of responder vs tested animals are indicated in bold above the columns. Columns represent the mean ± SEM of three determinations.
Hierarchy of HLA-A*0201-restricted HBV-specific cytotoxic T cell responses after DNA-based immunization

The pCMV-S2.S plasmid DNA was injected into regenerating muscle, a condition previously found to be optimal for the induction of CTL responses (20). Spleens were harvested 3 wk after DNA-based immunization, and splenocytes were stimulated in vitro with LPS blasts loaded with the different peptides to optimize peptide presentation. Fig. 2 summarizes data from CTL responses generated by DNA-based immunization and following a single in vitro peptide stimulation. As observed with individual peptide immunization, DNA injection generated cytotoxic responses specific for all HLA-A2 epitopes tested. Different peptide response profiles were observed. Responses specific for two epitopes (HBs183–191 and HBs348–357) were simultaneously induced in all DNA-immunized mice (19 and 12 animals tested, respectively). Peptide epitopes (HBs183–191 and HBs348–357) that was also observed after detected cytotoxic responses against the two superdominant separately. As shown in Fig. 3 for four animals, in each mouse we formed secondary in vitro restimulation using all peptides
gle in vitro stimulation was not always sufficient. Thus, we per-
To assay the individual CTL responses to the eight HLA-A2-re-
determinations.

Columns represent the mean
mice vs tested mice is indicated above each panel.

(number of responder mice. Interestingly, IFN-γ-secreting T cells after DNA-based immunization, we performed anti-

IFN-γ ELISPOT assays 3 wk after immunization. The number of epitope-specific CD8+ T cells was evaluated by measuring the number of IFN-γ-secreting cells in response to a short term stimulation of splenocytes with the eight epitope peptides tested separately.

The cytotoxic response is multiepitopic in DNA-immunized individual animals

To assay the individual CTL responses to the eight HLA-A2-re-
stricted epitopes, the number of effector cells available after a single in vitro stimulation was not always sufficient. Thus, we performed secondary in vitro restimulation using all peptides separately. As shown in Fig. 3 for four animals, in each mouse we detected cytotoxic responses against the two superdominant epitopes (HBs183–191 and HBs348–357) that was also observed after a single in vitro stimulation. Moreover, we detected simultaneous responses against three to six epitopes per mouse, although the pattern of peptide recognition varied from one animal to another. After two in vitro stimulations cytotoxic responses were detected more frequently and with increased cytolysis activity against the weaker epitopes, such as peptides HBs177–185, HBs204–212, HBs251–259, HBs260–269, and HBs335–343 were also poorly immunogenic after peptide immunization. Thus, following DNA-based immunization, processing of the HBV envelope protein leads to an immunohierarchy in CD8+ T cell responses, in which two superdominant epitopes can be defined (HBs183–191 and HBs348–357).

The cytotoxic response is multiepitopic in DNA-immunized individual animals

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FIGURE 2. CTL response against HBV-HLA-A*0201 epitopes after DNA-based immunization.

Three weeks after pCMV-S2.S injection, the cytotoxic activity of effector cells was measured against that of RNA-S HHD cells pulsed either with HBsAg peptides or HBc18–27 control peptide. Specific lysis was calculated as described in Fig. 1 and was considered positive when >10% at an E:T cell ratio of 100:1. Panels represent the specific lysis for an E:T cell ratio of 100:1 in 5–19 mice tested in independent experiments against the same epitope. The number of responder mice vs tested mice is indicated above each panel. Columns represent the mean ± SEM of three determinations.
epitope bound to the chimeric HHD monochain molecule. This was compared with the lysis mediated by the same effector cells against the HLA-A*0201-transfected P815 targets that present peptide associated with a human heterodimeric HLA-A*0201 molecule. Target cells presenting peptides on chimeric or on native HLA molecules were lysed with comparable efficiency by the effector cells (Fig. 5). This indicates that the effector cells generated in HHD mice were able to recognize epitopes in the native human HLA-A*0201 context, confirming that HHD mice are a relevant animal model for studying HLA-A*0201-restricted responses. Since murine CD8 molecules do not bind the \( \alpha_3 \) domain of human MHC class I molecules, this suggests that effector cells specific for peptide HBs183–191 and peptide HBs 347–358 generated after DNA-based immunization do not require CD8 involvement for a functional recognition between TCR and the peptide/MHC complex (Fig. 5, right).

HLA-A*0201 mouse CD8\(^+\) T cells induced after DNA-based immunization recognize endogenously synthesized HBV envelope proteins

Genetic immunization in HHD mice was shown to induce HLA-A2-restricted CTL responses specific for previously described HBV epitopes using peptides directly loaded onto target cells. To further characterize this response we performed an ELISPOT assay to monitor the T cell response to endogenously processed Ag. Splenocytes were infected with recombinant vaccinia virus encoding the three HBV envelope proteins carrying the HBsAg (rVV-S1.S2.S). The use of recombinant vaccinia virus expressing the large envelope protein leads to cellular accumulation of HBsAg without secretion of HBsAg particles (33). Hence, this system avoids presentation of secreted HBsAg to CD4\(^+\) T cells by APC. As shown in Fig. 6A, splenocytes from DNA-immunized mice secreting IFN-\( \gamma \) in response to stimulation with rVV-S1.S2.S-infected cells presenting endogenously processed HBV peptides. The secretion of IFN-\( \gamma \) was not due to vaccinia virus infection alone, as the background level of splenocytes infected with wild-type vaccinia virus never exceeded 15 SFC/10\(^6\) cells. To determine which cell type is involved in IFN-\( \gamma \) secretion we simultaneously performed an ELISPOT assay with CD8\(^+\) T cell-depleted splenocytes. The number of SFC specific for rVV-S1.S2.S-infected cells was dramatically decreased in the CD8\(^+\) T-depleted population (Fig. 6A), indicating that secretion of IFN-\( \gamma \) was due to recognition of the epitope/HLA-A2 complex by CD8\(^+\) T cells only. As a control for cell functionality after magnetic depletion, an ELISPOT assay using a pre-S2-specific T helper peptide was performed (Fig. 6B). The number of IFN-\( \gamma \)-secreting CD4\(^+\) T cells did not differ before and after CD8\(^+\) T cell depletion. These results show that

![Figure 3](https://example.com/fig3.png)  
**FIGURE 3.** Multiepitopic CTL response against HBV-HLA-A*0201 epitopes in individual animals. Four HHD mice (A, B, C, and D) were injected with 100 \( \mu \)g of pCMV-S2.S plasmid DNA encoding the HBsAg. Three weeks later, splenocytes were harvested and restimulated independently with LPS blasts loaded with the eight different HBsAg-HLA-A*0201 peptides. After two rounds of in vitro stimulation, the cytolytic activity was measured as described in Fig. 1. Specific lysis is given for four E:T cell ratios: 60:1, 20:1, 7:1, and 2:1 (black, dark gray, light gray, and white columns, respectively). Columns represent the mean \( \pm \) SEM of three determinations.

![Figure 4](https://example.com/fig4.png)  
**FIGURE 4.** Frequencies of epitope-specific IFN-\( \gamma \)-secreting cells in mouse splenocytes after DNA-based immunization. Total splenocytes from 10 pCMV-S2.S-immunized HHD mice were collected 3 wk after injection of plasmid and cultured in the presence of 1 \( \mu \)g/ml of HLA-A*0201 peptide during 40 h. IFN-\( \gamma \)-secreting cells were evaluated in an anti-IFN-\( \gamma \) ELISPOT assay. Background level was measured in wells containing splenocytes in medium only. The number of peptide-specific spots was obtained by subtracting the background from the number of spots appearing after HLA-A2 peptide stimulation. The results \( \pm \) SEM are given for 1000 CD8\(^+\) T cells, as determined by FACS analysis.
HLA-A2-restricted epitopes derived from the processing of endo-
HBV-derived epitopes. Using DNA-based immunization, CTL
HHD T cell repertoire is wide enough to recognize these eight
cytotoxic T cells displaying a broad specificity. CTL generated in
vector encoding HBV envelope proteins gives rise to high levels of
these mice we have shown that DNA-based immunization with a
viral (26) or anti-tumoral (25) immunotherapeutic strategies. Using
resent a versatile animal model for preclinical evaluation of anti-
1
CD8
T cells in HHD mice, and that the
HLA-A2-restricted responses are
strong cytotoxic responses in different animal models. However,
cellular immune responses have usually been characterized in mice
by examining a single immunodominant CTL epitope (18, 34). In
the model of influenza, it has been shown that DNA-based immu-
ization could lead to cytotoxic responses against subdominant
epitopes only when the dominant epitope was mutated (35). Few
studies have reported multispecific CTL responses, but these were
performed using polypepitope DNA vaccines (25, 36, 37) rather
than plasmid encoding a full-length antigenic protein (38, 39). In
this report we have analyzed the HLA-A2-restricted responses
against eight epitopes derived from the small HBV envelope protein.

Using peptide immunization, we have first shown that eight im-
munodominant epitopes derived from the HBV envelope protein
are presented by the HLA-A2 chimeric molecule and that they
stimulate CTL induction in HHD mice. These results indicate that
the epitopes defined in the HLA-A2 context in humans are also
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recognized by mouse CD8+ T cells in HHD mice, and that
the HHD T cell repertoire is wide enough to recognize these eight
HBV-derived epitopes. Using DNA-based immunization, CTL
against all epitopes carried by the encoded proteins were also in-
duced, albeit with differing frequencies. We further found that a
multispecific cytotoxic response can be simultaneously induced in
a single animal, and that the response to superdominant epitopes
(40) does not dampen the response to less dominant ones. In our
study superdominant epitopes are defined by the detection of
highly frequent specific cytotoxic CD8+ T cells in all immunized
mice. On the other hand, responses specific for less dominant
epitopes were sporadic, and specific CD8+ T cells were detected
with lower frequencies. This could be related to the fact that the
abundance of a given clone in a naive repertoire varies from mouse
to mouse. In certain animals, especially for weak epitopes, such a
clonal may be under the threshold required to encounter its cognate
Ag and to be activated (41). Interestingly, responses to subdomi-
nant epitopes can coexist with responses against superdominant
epitopes. This could have important implications for the generation
or the broadening of immune responses and for counteracting CTL
escape mutations. This is in accordance with what was observed in
acutely infected patients, for which CD8+ T cell responses are strong,
polyclonal, and specific for several epitopes derived from a single
protein. It was shown that CD8+ T cells specific for four different
HBV envelope epitopes could coexist in a given patient (24).

It was previously shown that the peptide hierarchy in T cell
responses does not strictly correlate with binding affinity to the
restriction element (42). Similarly, in our DNA-based immu-
ization study, the T cell peptide hierarchy did not correlate with the
affinity of peptides for the HLA-A2 class I molecule, suggesting
that competition for peptide fixation is not a major factor affecting
immunodominance. DNA immunization requires protein process-
ing and peptide transport by TAP molecules to achieve peptide
presentation and CTL induction. Thus, after genetic immunization
the weaker responses observed against some epitopes previously
described in HBV-infected humans during either the acute or the
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after two rounds of in vitro stimulation the responses specific for the weaker epitopes were confirmed by a higher number of mice recognizing these epitopes and by an increase in T cell cytotoxic activity. This confirms the relevance of the low responses against weak epitopes that could also be due to the limited number of CD8+ T cells in these Tg mice that never exceeded 5%. Moreover, in humans only CD8+ T cells specific for dominant epitopes (HBs335–343 or HBc18–27) have been detected ex vivo by tetramer staining without amplification (45). In contrast, detection of cytotoxic activity specific for less dominant epitopes required in vitro stimulation of T cells during at least 2 wk and in the presence of cytokines (24).

CTLs specific for the two superdominant epitopes (HBs183–191 and HBs335–343) were very frequent in DNA-immunized HHD mice. During acute HBV infection in humans the cytotoxic response is strong, polyclonal, and multipotent and is associated with viral clearance (46, 47). In self-limited acute infection several epitopes, notably HBC16–25, HBs183–191, and HBs335–343, are recognized by the majority of infected patients (4, 45). The frequency of HBV-specific CD8+ T cells detected by tetramer staining in acutely infected patients varied from 0.4 to 1.2/1000 CD8+ T cells depending on the epitope and the patient (45). In addition, in uninfected but intradermally HBsAg-vaccinated individuals, CD8+ T cell responses were measured by ex vivo ELISPOT assay, with frequencies for HLA-A2-restricted envelope epitopes ranging from 0.3 to 1.2/1000 CD8+ T cells (48). The epitope HBs183–191, shown here in HHD DNA-immunized mice to be superdominant, belongs to the group of peptides most frequently recognized by CD8+ T cells from acutely infected patients, and its frequency in mice (1/1000 CD8+ T cells) is comparable to that observed in acutely infected patients. Regarding the epitope HBs335–343 frequently recognized in humans, DNA-based immunization did not induce a strong CD8+ T cell response against this epitope, although specific T cells were present in HHD mice, as demonstrated by peptide injection. The weak response to HBs335–343 was also observed in another model of HLA-A2.1 mice using polypeptidic DNA constructs (49), suggesting a defect in Ag processing or transport. It has been shown that the proteasome may in some cases destroy the epitope by cleavage (50) and that TAP molecules do not transport all peptide precursors into the endoplasmic reticulum (51). Also, we cannot exclude that the cytokine environment could differ after virus infection or genetic immunization. It has been shown that in an inflammatory condition the constitutive proteasome is modified into immunoproteasome and that the epitope processing could be affected by either favoring or dampening epitope production (52). In addition, an alternative processing pathway is available for specific CTL induction by HBsAg, since exogenous envelope Ag can also enter the class I processing pathway and induce class I-restricted CTL (53, 54). It was also shown that the alternative exogenous or the classical endogenous pathway may generate different peptides in mice (54). The processing of HBsAg secreted following natural infection or produced after DNA-based immunization could thus result in slightly different sets of epitopes.

When stimulated in vitro, the CTL induced in HHD mice produced IFN-γ after recognition of endogenously processed peptides. Experiments in HBV transgenic mice have provided evidence for the roles of IFN-γ and TNF-α in the antiviral response to HBV (5, 17). In humans a predominance of Th0 cells was detected in the liver, thus favoring the persistence of the virus (55, 56). In contrast to what was observed during chronic hepatitis, a strong IFN-γ production by Th1 cells in the peripheral blood was detected following antigenic stimulation of PBMC from acutely infected patients (57). Thus, it is tempting to speculate that IFN-γ-secreting T cells induced after DNA-based immunization could facilitate eradication of HBV infection in the liver.

Our studies show that DNA-based immunization of HHD mice can effectively induce CTL responses comparable to those against HLA-A2-restricted epitopes observed in HBV-infected patients. This suggests that the pathways of Ag processing and presentation for MHC class I-restricted CTL recognition of viral proteins expressed after DNA immunization mimic those previously described for viral infection, and that DNA-induced CTL could have the same immune potential as those developing during natural infection in human patients.

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References

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