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

Delphine Loirat,^{1*†} François A. Lemonnier,[‡] and Marie-Louise Michel^{2*}

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 (β_2 -microglobulin^{-/-} D^b^{-/-}) and transgenic for a chimeric HLA-A*0201/D^b molecule covalently bound to the human β_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- γ -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)³ 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- γ , IFN- α , IFN- β , and TNF- α) 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- α 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

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³ Abbreviations used in this paper: HBV, hepatitis B virus; HBsAg, hepatitis B surface Ag; HBcAg, hepatitis B core antigen; β_2 m, β_2 -microglobulin; CM, culture medium; ELISPOT, enzyme-linked immunospot; MOI, multiplicity of infection; rVV, recombinant vaccinia virus; SFC, spot-forming cells; HHD, Human Human D^b.

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 Human D^b (HHD) mice derived from a strain deficient for the β_2 -microglobulin (β_2m) gene and the mouse MHC class-I H-2D^b molecules and transgenic for a chimeric HLA-A*0201/D^b 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^{+/+} $\beta_2m^{-/-}$ D^b^{-/-} mice used for immunization were 6–8 wk old. The $\beta_2m^{-/-}$ D^b^{-/-} strain was made transgenic for a chimeric HLA-A*0201 monochain, the HHD molecule (23) in which the human β_2m molecule is covalently linked to the NH₂ terminus of a hybrid MHC class I heavy chain. The hybrid heavy chain is composed of the $\alpha 1$ and $\alpha 2$ domains of the human HLA-A*0201 MHC class I molecule and the $\alpha 3$, transmembrane, and cytoplasmic domains of the mouse H-2D^b MHC class I molecule. The expression of this monomeric MHC class I molecule on the cell surfaces in β_2m - and H-2D^b-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 μ g of HLA-A*0201-restricted peptide combined with 140 μ g of T helper peptide (hepatitis B nucleocapside, HBc_{128–140} TPPATRPNPAPIL) emulsified in IFA (Difco, Detroit, MI). The emulsion (100 μ l) was injected s.c. at the base of the tail (27). For loading LPS blasts and target cells, peptides were dissolved in RPMI-20% DMSO at 1 mg/ml, aliquoted, and stored at -20°C .

Peptide binding and stabilization of HLA-A*0201 molecules

T2 cells (TAP⁻, HLA-A*0201⁺) were incubated overnight at 37°C in serum-free RPMI medium supplemented with 100 ng/ml human β_2m (Sigma, St. Louis, MO) in the absence or the presence of HLA-A*0201-tested peptides at various final concentrations (10, 1, and 0.1 μ M). Subsequently, the level of HLA-A*0201 molecules stabilized in the presence of a given peptide was evaluated by FACS analysis. Briefly, T2 cells were incubated for 20 min at 4°C with an anti-HLA-A*0201 mAb (BB7.2), washed twice, incubated with FITC-conjugated anti-mouse IgG (Diagnostic Pasteur, Marnes la Coquette, France), washed twice, incubated in PBS containing 1/1000 propidium iodide, and analyzed using a FACSCalibur cytofluorometer (Becton Dickinson, San Jose, CA). The mean intensity of fluorescence observed for each peptide concentration after subtraction of the mean intensity of fluorescence observed without peptide was used as an estimate of peptide binding. A peptide derived from the hepatitis B nucleocapside HBc_{18–27} (FLPSFFPSV) was used as a positive control to define the maximal binding (27). For each peptide, the concentration needed to reach 20% of maximal binding was calculated. RA is the ratio of the concentration of tested peptide and that of HBc_{18–27} reference peptide needed to reach this value (25).

HBsAg expression vector and DNA-based immunization

The pCMV-S2.S plasmid contains part of the envelope-coding domain of the HBV genome. In this plasmid (14) the pre-S2 and S HBV domains were placed under transcriptional control of the human CMV immediate early gene promoter, allowing the expression of the HBV small and the middle envelope proteins, both carrying the HBsAg. Plasmid DNA used for in vivo immunization was prepared using Qiagen DNA purification columns (Endofree Plasmid Kit; Qiagen, Hilden, Germany). For DNA-based immunization, HBsAg expression vector pCMV-S2.S was injected directly into regenerating tibialis anterior muscles as previously described

(13, 28). Each muscle was injected with 50 μ l of DNA at 1 mg/ml in PBS, such that each animal received a total of 100 μ g 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 μ g/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 P815 cells were transfected with the native HLA-A*0201 and human β_2m 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^{-5} M 2-ME, 1 mM sodium pyruvate, LPS from *Salmonella typhosis* (25 μ g/ml; Sigma, St. Louis, MO), and dextran sulfate (7 μ g/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 μ g/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 (5×10^6 cells in 24-well plates) in α -MEM (Life Technologies) supplemented with 10 mM HEPES, nonessential amino acids, 1 mM sodium pyruvate, 5×10^{-5} M 2-ME, antibiotics, and 10% FCS (Myoclon, 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 μ g/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 (HBc_{18–27}) as a negative control. After a 4-h incubation at 37°C, supernatants were collected and counted on a beta counter. Spontaneous 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)] \times 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-infected cell stimulation by cytokine-specific ELISPOT assay. Ninety-six-well nitrocellulose-backed plates (Multiscreen; Millipore, Molsheim, France) were coated with 50 μ l of mouse IFN- γ mAb (5 μ g/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 (1×10^6 /well) were incubated for 40 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 μ g/ml; see Table I) or a mouse MHC class II-restricted peptide (HBs_{126–138}) derived from the pre-S2 domain of the HBV envelope middle protein (3 μ g/ml) (17) or were infected with a recombinant vaccinia virus encoding the three HBV envelope proteins (rVV S1.S2.S) (29) at a multiplicity of infection (MOI) of 1/1 (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 μ g/ml) to the splenocytes. After incubation, cells were lysed with water for 4 min, and the wells were washed three times each with PBS-Tween or PBS alone 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-4,3-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

Table I. HLA-A0201 peptide-binding and stabilizing capacities of HBV envelope-derived epitopes

Position ^a	Amino Acid Sequence ^b	RA ^c	Ref. ^d
177–185	VLQAGFFLL	441.3	3
183–191	FLLTRILTI	14.28 ± 2.98	3
204–212	FLGGTPVCL	4.07	3
251–259	LLCLIFLLV	>450	24
260–269	LLDYQGMLP	1.68 ± 0.02	24
335–343	WLSLLVPFV	1.41 ± 0.11	24
348–357	GLSPTVWLS	1.12 ± 0.12	24
370–379	SIVSPFIPLL	5.27 ± 0.7	3

^a 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.

^b Amino acid sequence of the peptides. Amino acids involved in the binding to the HLA-A*0201 molecule are in bold.

^c 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*).

^d Reference corresponding to the first description of a cytotoxic response specific for the epitope in HBV-infected patients.

were counted in a double-blinded fashion as single spot-forming cells (SFC) under a stereo binocular. The number of Ag-specific IFN- γ -secreting CD8⁺ T cells was determined by subtracting the number of background spots from the number of spots obtained 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 Biotec, 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

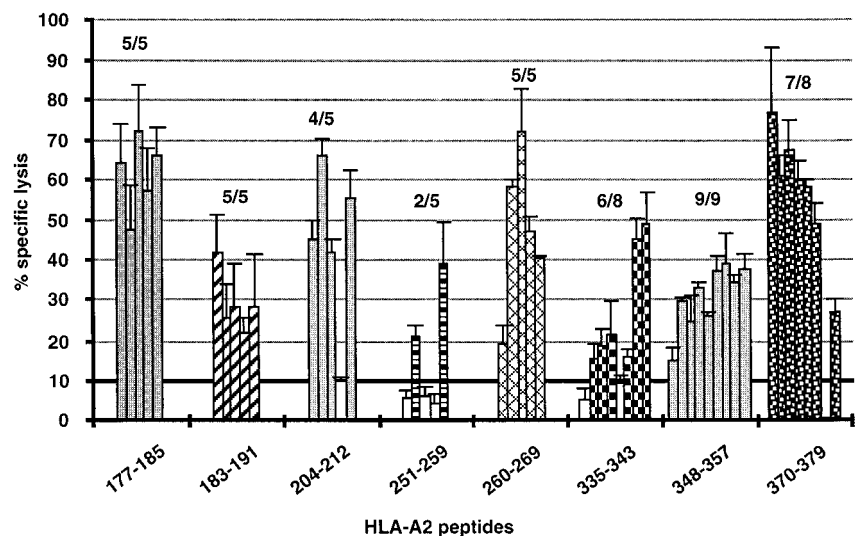
We have analyzed the capacity of eight HBV-envelope HLA-A*0201 peptides described as epitopes in humans to bind and stabilize the HLA-A*0201 molecule on the T2 cell surface. T2 cells are HLA-A*0201 positive and TAP-negative human cells expressing unstable HLA-A*0201 molecules at 37°C. Providing exogenous 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 after comparison with a reference peptide (HBc_{18–27}) 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 (HBs_{260–269}, HBs_{335–343}, HBs_{348–357}), intermediate (HBs_{183–191}, HBs_{204–212}, HBs_{370–379}), and very low (HBs_{177–185} and HBs_{251–259}) 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/epitope complex was well recognized by TCR. Most peptides (HBs_{177–185}, HBs_{183–191}, HBs_{204–212}, HBs_{260–269}, HBs_{348–357}, and HBs_{370–379}) induced a strong cytotoxic response in almost all immunized mice, whereas two peptides (HBs_{251–259} and HBs_{335–343}) induced a weaker response in some injected mice. However, the weak response induced by injection of the peptide HBs_{251–259} 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 HBs_{335–343}, 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.

FIGURE 1. CTL response against HLA-A*0201-restricted HBV envelope epitopes after single peptide immunization. The cytotoxic activity of effector 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 $\geq 10\%$ 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 \pm 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). Splensens 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 HBs₃₇₀₋₃₇₉ had an intermediate pattern of recognition, as 5 of 10 animals tested developed a specific cytotoxic response. Responses specific for the other epitopes (HBs₁₇₇₋₁₈₅, HBs₂₀₄₋₂₁₂, HBs₂₅₁₋₂₅₉, HBs₂₆₀₋₂₆₉, and HBs₃₃₅₋₃₄₃) were sporadic. Interestingly, peptides HBs₂₅₁₋₂₅₉ 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

To assay the individual CTL responses to the eight HLA-A2-restricted 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 cytolytic activity against the weaker epitopes, such as peptides HBs₁₇₇₋₁₈₅ and HBs₂₀₄₋₂₁₂ (Fig. 3A), HBs₃₇₀₋₃₇₉ (Fig. 3, A, B, and D) and HBs₂₆₀₋₂₆₉ (Fig. 3C). These results confirmed the significance of responses against weak epitopes that were obtained at low frequency following 1 wk of *in vitro* stimulation. Moreover, we never observed *in vitro* CTL induction from mice either unimmunized or immunized with an

irrelevant vector (pCMV-*lacZ*; data not shown). Thus, responses generated against the superdominant epitopes do not inhibit responses against weaker epitopes.

Frequencies of IFN- γ -secreting CD8⁺ T cells after DNA-based immunization

To evaluate the frequency of HBV-specific CD8⁺ T cells present in the spleen 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 epitopic peptides tested separately. The high frequency of T cells specific for peptide HBs₃₄₈₋₃₅₇ and peptide HBs₁₈₃₋₁₉₁ was found in all animals tested, and T cells specific for peptide HBs₃₇₀₋₃₇₉ were found in 2 of 10 mice tested (Fig. 4). The mean of CD8⁺ T cells specific for peptide HBs₃₄₈₋₃₅₇ was 2-fold higher than that for peptide HBs₁₈₃₋₁₉₁ (2/1000 and 1/1000 of total CD8⁺ T cells, respectively). The number of T cells specific for peptide HBs₃₇₀₋₃₇₉ was 0.15 and 0.5/1000 CD8⁺ for two responder mice. Interestingly, IFN- γ -secreting T cells detected by the ELISPOT assay were mainly specific for the two peptides described as superdominant in the CTL assay after *in vitro* stimulation (see Figs. 2 and 3), but not for the weak CTL epitopes. In addition, we never detected any IFN- γ -secreting T cells for an irrelevant control peptide (Hbc₁₈₋₂₇). Detection of cytotoxic activity was performed 1 wk after *in vitro* stimulation in the presence of peptide-pulsed LPS blasts as APCs, a condition that favors the amplification of CD8⁺ T cells by up to 10-fold, as tested by FACS analysis (data not shown). In contrast, the ELISPOT assay was performed *ex vivo* following short term restimulation. In similar experimental conditions it has been shown that only a fraction of perforin-positive T cells produce IFN- γ (32). Thus, the precursor frequency of the CD8⁺ T cells specific for the weak epitopes was probably too low to be detected in this short term stimulation assay.

CTL educated in HHD mice recognize epitopes presented by the native human HLA-A2 molecule

To fully characterize the cytotoxic response generated after DNA-based immunization, we evaluated the cytotoxic activity of effector cells toward target cells expressing the chimeric or the native HLA-A*0201 molecule. We used effector cells specific for the two most frequently recognized epitopes (HBs₁₈₃₋₁₉₁ and HBs₃₄₈₋₃₅₇) derived from mice after a single DNA injection and a single *in vitro* peptide stimulation. HHD-transfected HeLa cells were used as targets to determine the level of lysis against cells presenting the

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 RMA-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 \pm SEM of three determinations.

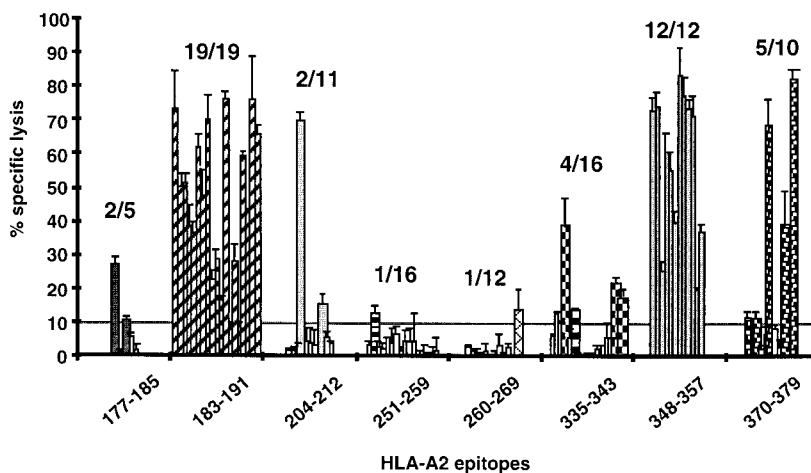
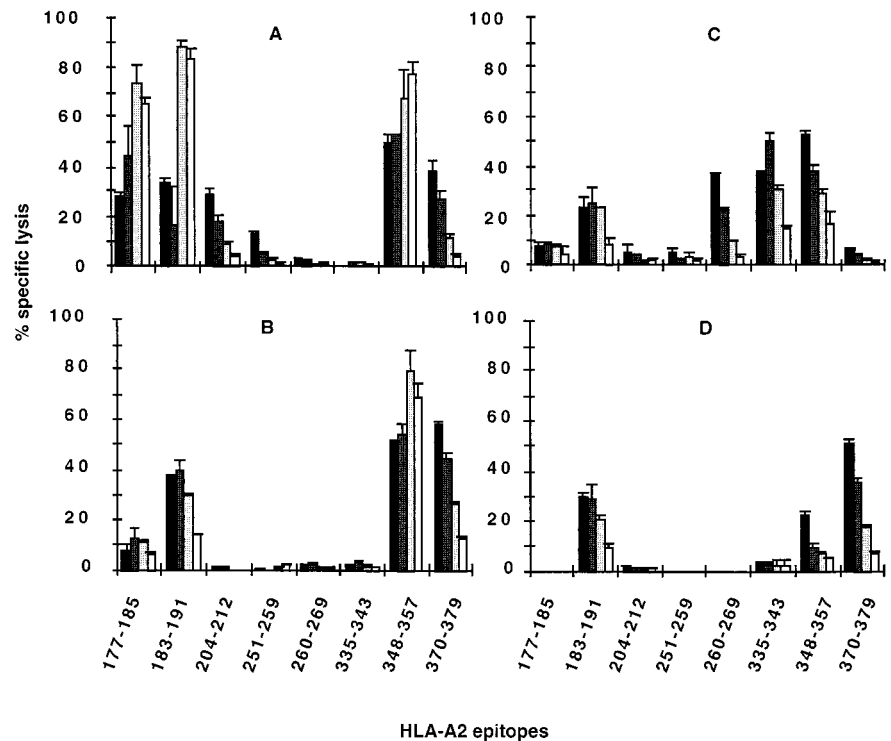


FIGURE 3. Multi-epitopic CTL response against HBV-HLA-A*0201 epitopes in individual animals. Four HHD mice (A, B, C, and D) were injected with 100 μ g of pCMV-S2.S plasmid DNA encoding the HBsAg. Three weeks later, splenocytes were harvested and restimulated independently with LPS blasts loaded with the eight different HBsAg-HLA-A*0201 peptides. After two rounds of in vitro stimulation, the cytotoxic 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.



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 α 3 domain of human MHC class I molecules, this suggests that effector cells specific for peptide HBs₁₈₃₋₁₉₁ and peptide HBs₃₄₇₋₃₅₈ 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 secrete IFN- γ in response to stimulation with rVV-S1.S2.S-infected cells presenting endogenously processed HBV peptides. The secretion of IFN- γ was not due to vaccinia virus infection alone, as the background level of splenocytes infected with wild-

type vaccinia virus never exceeded 15 SFC/10⁶ cells. To determine which cell type is involved in IFN- γ 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- γ 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- γ -secreting CD4⁺ T cells did not differ before and after CD8⁺ T cell depletion. These results show that

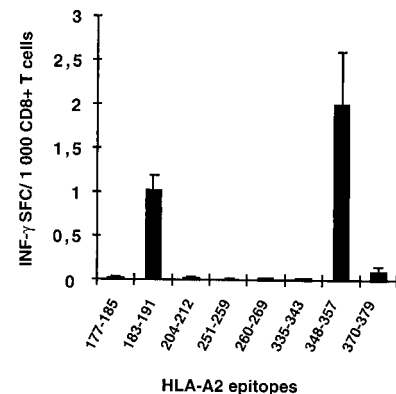


FIGURE 4. Frequencies of epitope-specific IFN- γ -secreting cells in mouse splenocytes after DNA-based immunization. Total splenocytes from 10 pCMV-S2.S-immunized HHD mice were collected 3 wk after injection of plasmid and cultured in the presence of 1 μ g/ml of HLA-A*0201 peptide during 40 h. IFN- γ -releasing cells were evaluated in an anti-IFN- γ 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.

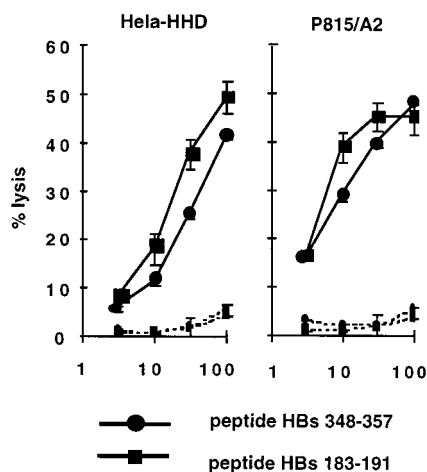


FIGURE 5. Recognition of peptides bind to the native HLA-A*201 molecule by HHD-CTL. Effector cells were obtained by culturing splenocytes from a DNA-based immunized mouse for 1 wk with LPS blasts loaded with HBs₃₄₈₋₃₅₇ (●) or HBs₁₈₃₋₁₉₁ (■) as APC. Cytotoxic activity was evaluated on HeLa-HHD (*left*) and on P815/A2 (*right*) target cells expressing the chimeric (HHD) or the wild-type HLA-A*201 molecules, respectively. Targets were peptide loaded (continuous lines) or unloaded (dotted lines) cells. Three determinations of cytotoxic activity were performed, and SEMs are given. Results are shown for one representative mouse.

CD8⁺ T cells induced after pCMV-S2.S immunization recognize HLA-A2-restricted epitopes derived from the processing of endogenously produced HBV envelope proteins.

Discussion

Mouse MHC class I knockout, HLA-A2 transgenic mice (23) represent a versatile animal model for preclinical evaluation of antiviral (26) or anti-tumoral (25) immunotherapeutic strategies. Using these mice we have shown that DNA-based immunization with a vector encoding HBV envelope proteins gives rise to high levels of cytotoxic T cells displaying a broad specificity. CTL generated in HHD transgenic mice secrete IFN- γ following antigenic stimulation and recognize eight epitopes that have been previously described in HBV-infected humans during either the acute or the resolution phase of the disease.

Genetic immunization is a powerful tool for the induction of 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 immunization 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 polyepitopic 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 immunodominant 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 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

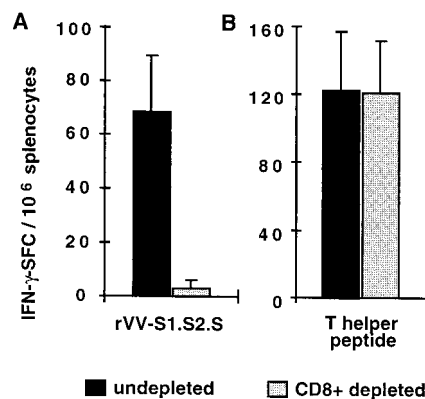


FIGURE 6. IFN- γ -secreting T cells after DNA-based immunization. IFN- γ -releasing cells were evaluated in an anti-IFN- γ ELISPOT assay performed on whole (■) or on CD8⁺ T cell-depleted (▨) spleen populations 3 wk after injection of pCMV-S2.S plasmid. Splenocytes were infected with recombinant vaccinia virus (rVV-S1.S2.S) encoding the three HBV envelope proteins (A) or cultured in the presence of pre-S2-derived T helper peptide (B). Background levels were measured, respectively, after infection of splenocytes with wild-type vaccinia virus or in wells containing splenocytes in medium only and were subtracted from the specific number of SFC. Results are given for the mean \pm SEM of three individual mice.

against all epitopes carried by the encoded proteins were also induced, 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 clone may be under the threshold required to encounter its cognate Ag and to be activated (41). Interestingly, responses to subdominant 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 immunization 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 processing 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 humans could result from a defect in processing or transport of the peptides in mice. It has been shown that mouse TAP transporters are less permissive than human TAP transporters (43). In addition, we cannot exclude a competition in APC recognition between CD8⁺ T cells specific for the superdominant epitopes and T cells specific for other epitopes (44). Nevertheless,

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 HBs₃₄₈₋₃₅₇) were very frequent in DNA-immunized HHD mice. During acute HBV infection in humans the cytotoxic response is strong, polyclonal, and multiepitopic and is associated with viral clearance (46, 47). In self-limited acute infection several epitopes, notably Hbc₁₈₋₂₇, 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 polyepitopic 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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