

# Enhanced In Vitro Potency and In Vivo Immunogenicity of a CTL Epitope from Hepatitis C Virus Core Protein following Amino Acid Replacement at Secondary HLA-A2.1 Binding Positions

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## Abstract

Since the natural immune response to hepatitis C virus (HCV) is often unable to clear the infection, to enhance immunogenicity we studied substituted peptides from an HCV cytotoxic T lymphocyte (CTL) epitope (C7A2) from a conserved region of the HCV core protein (DLMGYIPLV) recognized by CTL lines from HLA-A2.1<sup>+</sup> HCV-infected patients and HLA-A2.1 transgenic mice. HLA-A2.1 binding, human and murine CTL recognition, and in vivo immunogenicity (using mice transgenic for human HLA-A2 in lieu of immunizing humans) were analyzed to define peptides with enhanced immunogenicity. Peptides substituted at position 1 showed enhanced HLA-A2 binding affinity, but paradoxically poorer immunogenicity. A peptide with Ala substituted at position 8 (8A) showed higher HLA-A2 binding affinity and CTL recognition and was a more potent in vivo immunogen in HLA-A2-transgenic mice, inducing higher CTL responses with higher avidity against native C7A2 than induced by C7A2 itself. These results suggest that peptide 8A is a more potent in vitro antigen and in vivo immunogen than C7A2 and may be useful as a vaccine component. They provide proof of principle that the strategy of epitope enhancement can enhance immunogenicity of a CTL epitope recognized by human CTL. (*J. Clin. Invest.* 1998. 102:1239–1248.) Key words: cytotoxic T lymphocyte • epitope enhancement • major histocompatibility complex class I affinity • peptide, synthetic • vaccine

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## Introduction

Hepatitis C virus (HCV)<sup>1</sup> is a single-stranded RNA virus responsible for the majority of non-A non-B hepatitis (1). Infection by HCV frequently evolves to chronicity, and in many cases leads to liver cirrhosis and hepatocellular carcinoma (2). The cellular immune response is thought to be responsible for viral clearance in many viral infections (3–7), and in the case of HCV, a cytotoxic T lymphocyte (CTL) response is present in acutely and chronically infected patients (8–14), but its role in viral clearance has not been elucidated. CTL responses have been detected in PBMCs and intrahepatic lymphocytic infiltrate in patients with chronic hepatitis (15) and in the liver of infected chimpanzees (16), suggesting that in these cases the virus is able to persist despite this immune response (17). However, the CTL response might contribute to the immunopathogenesis observed during the course of infection. The reasons for the inadequacy of this immune response in chronically infected patients are not known (18), but efforts to improve antiviral mechanisms might lead to improved viral clearance and recovery from infection.

CD8<sup>+</sup> CTLs recognize antigens as peptides presented by class I molecules of the major histocompatibility complex (MHC) on the cell surface. These peptides are usually 8–10 amino acids long and are generated after processing of intracellular antigens (3, 19–21). Analysis of peptides presented by MHC class I molecules has led to the definition of several sequence patterns or motifs (22–24) for peptides that bind to each particular MHC allele or group of alleles (supermotif) (25). These motifs are based on the presence in precise positions in the peptide sequence of several amino acids (agretopic residues) called anchor residues (22, 26), responsible for interactions between peptide and MHC molecules, as well as other secondary positions that may help in stabilizing these interactions (27–29). The use of these motifs to predict peptides able to bind to MHC molecules, together with the development of MHC-peptide binding assays, has led to the characterization of many CTL epitopes in the HCV polyprotein presented by different MHC molecules (9, 10, 12, 30). Among the best studied motifs is that of HLA-A2.1, which is prevalent in a high percentage of the population (31). Several reports describe the

1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; CTM, complete T cell medium; FI, fluorescence index; HCV, hepatitis C virus; IFA, incomplete Freund's adjuvant; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; TCR, T cell receptor.

binding motif for this allele, pointing out the importance of anchor as well as secondary residues. Also, MHC binding has been correlated with immunogenicity in different mouse and human systems (30, 32–37).

Despite the presence of the typical anchor residues, a peptide epitope may have different binding ability depending on the other secondary residues. Thus, the presence of some amino acids in secondary positions may enhance or impair its binding ability (28, 38, 39). However, some other amino acids (epitopic residues) are responsible for recognition by the T cell receptor (TCR). Thus, T cell response is triggered by interactions in the trimolecular complex MHC–antigenic peptide–TCR, together with some other costimulatory molecules (40, 41). These interactions occur between the antigenic peptide and pockets in the structure of both MHC and TCR molecules, and changes in the peptide sequence may affect any of these interfaces. Some of these changes have been described as having a negative effect in the T cell response, by impairing MHC binding or T cell recognition, but in other cases, replacement of amino acids at certain positions increased the peptide immunogenicity, either due to improvement of MHC binding affinity or T cell recognition (32–35, 38). Thus, our hypothesis is that appropriate modification of the sequence of a peptide epitope can increase the affinity for the MHC molecule without interfering with recognition by the TCR of T cells specific for the natural viral sequence. Therefore, by this process we call epitope enhancement, one should be able to create a more potent vaccine (42–45).

In this report, we analyze the CTL response against C7A2, a conserved peptide epitope from HCV core protein (amino acids 132–140), presented by HLA-A2.1 class I molecules, and recognized by CTLs from HCV chronically infected individuals (9, 12, 30, 46), implying that this epitope is naturally processed and presented in hepatitis C infection. This epitope has the typical HLA-A2.1 binding motif, but, despite the presence of L at position 2 and V at position 9, has an intermediate binding affinity (12, 30). Thus, we analyzed the role of the different peptide residues in HLA-A2.1 binding and TCR recognition to characterize the importance of these positions and the effect of replacement by different amino acids. Changes introduced in secondary HLA-A2.1 binding anchor positions showed that these peptides had a higher binding affinity than C7A2, and as a consequence, were more potent than C7A2 for recognition by C7A2-specific CTL in most cases, in accordance with previous results reporting that MHC class I binding affinity correlates with peptide potency (30, 32–37). Together with *in vitro* antigenicity, *in vivo* ability to induce an immune response has been tested with these peptides taking advantage of a transgenic mouse model expressing human HLA-A2.1 molecules (9, 47, 48) in lieu of immunizing humans. Immunogenicity in this transgenic mouse model has been shown to correlate with antigenicity for human CTL (9, 49). In this case, peptide 8A (a peptide with Ala substituted at position 8) was more immunogenic than native C7A2, whereas other peptides with good *in vitro* recognition did not induce any CTL activity. Peptide 8A-induced CTLs were more effective at lysing C7A2-pulsed cells than the CTLs induced by C7A2 itself. Moreover, CTLs induced in 8A-immunized mice recognized cells pulsed with sequences belonging to other viral genotypes. These results demonstrate that improvement of peptide epitope sequences by replacement of agretopic residues (epitope enhancement) is a good approach to enhance *in vitro*

and *in vivo* immunogenicity, and suggest that 8A is a more effective antigen to increase anti-HCV immune response for immunotherapy or vaccine prophylaxis. This study constitutes the first proof of principle by *in vivo* immunization that epitope enhancement can improve immunogenicity of a viral CTL epitope recognized by human CTLs.

## Methods

**Synthetic peptides.** Peptides were prepared in an automated multiple peptide synthesizer (Symphony; Protein Technologies, Inc.) using Fmoc chemistry. They were purified by reverse-phase HPLC, and their sequences were confirmed on an automated sequencer (477A; Applied Biosystems, Foster City, CA).

**Cells.** The T2 cell line, a gift of Peter Cresswell, is deficient in TAP1 and TAP2 transporter proteins and expresses low levels of HLA-A2.1 (50, 51). The human B lymphoblastoid cell line HMYC1R transfected with HLA-A2.1 (C1R.A2.1) was provided by William Biddison. Cell line C1R.AAD (HMYC1R transfected with the HLA chimeric molecule containing  $\alpha 1$  and  $\alpha 2$  domains from human HLA-A2.1 and  $\alpha 3$  from mouse H-2D<sup>d</sup>) has been previously described (48). Cell lines were maintained in complete T cell medium (CTM; 1:1 mixture of RPMI:EHAA; Life Technologies, Grand Island, NY) containing 10% FBS, 4 mM glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 50  $\mu$ M 2-mercaptoethanol.

**Human subjects.** PBMCs were isolated from a patient with chronic hepatitis C. The patient had anti-HCV-specific antibodies with the commercially available assay (Ortho Diagnostics, Raritan, NJ) and was HCV RNA positive by polymerase chain reaction (PCR).

**Mice.** Transgenic A2Kb mice (a gift of Linda Sherman, Scripps Research Institute, La Jolla, CA) and transgenic AAD mice, derived from breeders developed at the University of Virginia colony, were bred in our colony at BioCon Inc. (Rockville, MD). These animals have been previously described (48, 52) and they express  $\alpha 1$  and  $\alpha 2$  domains from the human HLA-A2.1 molecule and  $\alpha 3$  from mouse H-2K<sup>b</sup> and H-2D<sup>d</sup> molecules, respectively.

**Binding assays.** Peptide binding to HLA molecules was measured using the T2 mutant cell line according to a protocol previously described (53). T2 cells ( $3 \times 10^5$ /well) were incubated overnight in 96-well plates with culture medium (1:1 mixture of RPMI 1640/EHAA containing 2.5% FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin) with 10  $\mu$ g/ml  $\beta 2$ -microglobulin (Sigma Chemical Co., St. Louis, MO) and different peptide concentrations. The next day, cells were washed twice with cold PBS containing 2% FBS and incubated for 30 min at 4°C with anti-HLA-A2.1 BB7.2 mAb (1/80 dilution from hybridoma supernatant) and 5  $\mu$ g/ml FITC-labeled goat anti-mouse Ig (PharMingen, San Diego, CA). Cells were washed twice after each incubation, and HLA-A2.1 expression was measured by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA). HLA-A2.1 expression was quantified as fluorescence index (FI) according to the formula: FI = (mean fluorescence with peptide – mean fluorescence without peptide)/mean fluorescence without peptide. Background fluorescence without BB7.2 was subtracted for each individual value. To compare the different peptides, FI<sub>0.5</sub>, the peptide concentration that increases HLA-A2.1 expression by 50% over no peptide control background, was calculated from the titration curve for each peptide.

**CTL generation in humans.** CTL lines were generated according to the protocol previously described (12, 14). PBMCs from a patient chronically infected with HCV were separated by Ficoll-Hypaque and  $10^6$  cells were stimulated in 400  $\mu$ l in 48-well plates with  $2 \times 10^6$  autologous cells pulsed previously for 3 h with 10  $\mu$ M peptide in CTM. After 3 d, the same volume of CTM with IL-2 (10% vol/vol) was added to each well, and the cells were further expanded on day 6 with IL-2 containing CTM. This cycle was repeated every 10 days. For the first three times,  $2 \times 10^6$  peptide pulsed irradiated (3,000 rads) autologous PBMCs were used as antigen-presenting cells, and

in subsequent cycles, CTLs were stimulated with  $3 \times 10^5$  peptide pulsed irradiated (3,000 rads) autologous Concanavalin A (Con A) blasts and  $1.5 \times 10^6$  irradiated allogeneic PBMCs. Con A blasts were obtained by stimulating PBMCs with 10  $\mu\text{g/ml}$  Con A and expanding with CTM containing 50 U/ml IL-2 and 1 U/ml IL-6. Con A blasts were stimulated with Con A every 8–10 days.

**CTL generation in mice.** 8- to 12-wk-old mice were immunized subcutaneously in the base of the tail with 100  $\mu\text{l}$  of an emulsion containing 1:1 incomplete Freund's adjuvant (IFA) and PBS solution with antigens and cytokines (50 nmol CTL epitope, 50 nmol HBV core 128–140 helper epitope, 3  $\mu\text{g}$  of IL-12, and 3  $\mu\text{g}$  of granulocyte macrophage colony stimulating factor) (54). Mice were boosted 2 wk later, and spleens removed 10–14 d after the boost. Immune spleen cells ( $2.5 \times 10^6/\text{well}$ ) were stimulated in 24-well plates with autologous spleen cells ( $5 \times 10^6/\text{well}$ ) pulsed for 2 h with 10  $\mu\text{M}$  CTL epitope peptide in CTM with 10% T-Stim (Collaborative Biomedical Products, Bedford, MA). After two in vitro stimulations with peptide-pulsed syngeneic spleen cells, CTL lines were maintained by weekly restimulation of  $10^6$  CTL/well with  $2 \times 10^5$  peptide pulsed irradiated (10,000 rads) C1R.AAD cells and  $4 \times 10^6$  C57/Bl6 irradiated (3,000 rads) spleen cells as feeders.

**Cytotoxicity assay.** CTL activity was measured using a 4-h assay with  $^{51}\text{Cr}$ -labeled target cells. Target cells ( $10^6$ ) were pulsed in 100  $\mu\text{l}$  CTM with or without 10  $\mu\text{M}$  peptide and 150  $\mu\text{Ci}$   $^{51}\text{Cr}$  for 2 h, washed three times, and added to the plates containing different number of effector cells in a final volume of 200  $\mu\text{l}$ . In peptide titration assays, target cells were pulsed with  $^{51}\text{Cr}$  for 2 h, washed three times, and added to the plates with different peptide concentrations. Effector cells were added 2 h later, and the supernatants were harvested and counted after an additional 4 h of incubation. The percentage of specific  $^{51}\text{Cr}$  release was calculated as:  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ . Spontaneous release was determined from target cells incubated without effector cells, and maximum release was determined in the presence of 5% Triton X-100. C1R.A2.1 and C1R.AAD lines and AAD and A2Kb Con A blasts were used as targets. Con A blasts were prepared by culturing  $3 \times 10^6$  spleen cells in 2 ml of CTM in the presence of 2  $\mu\text{g/ml}$  of Con A in 24-well culture plates. After 2 days, cells were harvested and processed for labeling as described.

## Results

**C7A2 Ala-substituted peptides binding to HLA-A2.1 molecules.** A first approach to enhance peptide immunogenicity is to improve the affinity of CTL epitopes for HLA class I molecules (34, 35). For this reason, we decided to focus on a peptide from a conserved region from the HCV core protein (amino acids 132–140) designated C7A2, previously described as a CTL epitope by ourselves and others (8, 10). This epitope is endogenously processed and presented and recognized by HLA-A2.1-restricted CTL in HCV-infected patients (9, 10, 12), induces in vitro primary responses in normal donors (37, 55), is immunogenic in HLA-A2.1 expressing transgenic mice (9, 37), and has been described as a binder with intermediate affinity to HLA-A2.1 molecules (12, 30). This intermediate binding affinity allowed us to introduce modifications aimed at improving binding, compared with other HCV epitopes described as high affinity binders and more difficult to improve. It is also relatively conserved, with only a few conservative substitutions reported (56, 57). Such a conserved epitope may be more valuable in a vaccine than a higher affinity one that is more variable.

We evaluated the binding affinity of wild-type C7A2 (C7A2-WT) with a T2 binding assay (53), measuring the cell surface stabilization of HLA-A2.1 molecules after incubation

with peptide. To compare the different peptides,  $\text{FI}_{0.5}$  was chosen as a way to compare titrations of the peptides and relative affinity for MHC molecules. Using this method,  $\text{FI}_{0.5}$  of 10  $\mu\text{M}$  was calculated for C7A2-WT.

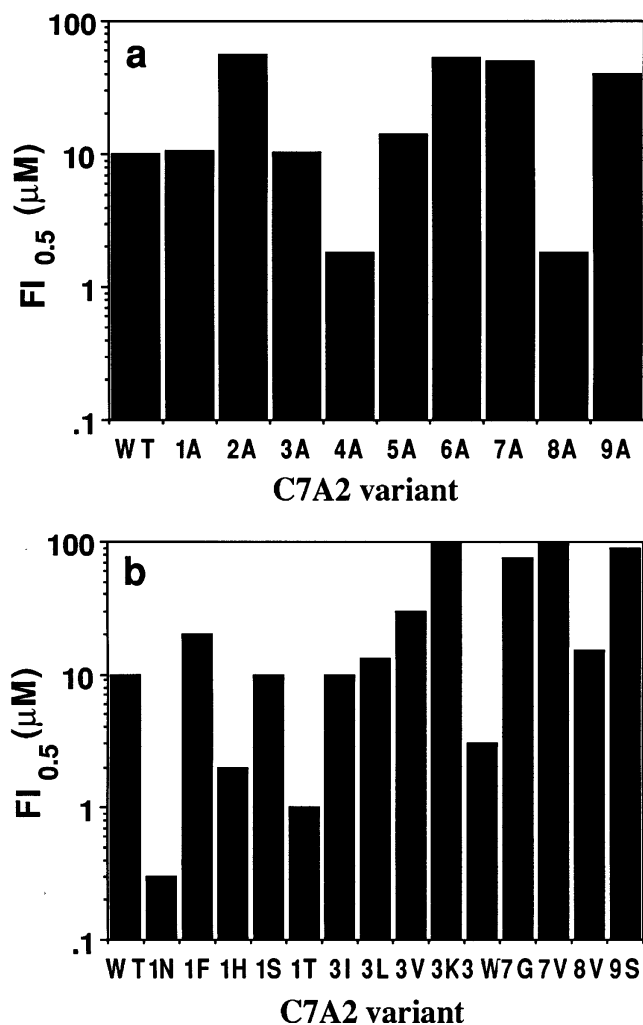
In a first set of experiments to define key functional residues, peptides with alanine substitutions at each one of the positions were synthesized and tested in binding assays (Table I). C7A2-WT has the typical motif for binding to HLA-A2.1, with L and V at positions 2 and 9, respectively (22, 23). Thus, substitutions at these positions would eliminate binding, but also substitutions in other positions might tell us the importance of other secondary residues. Binding experiments with these alanine-substituted peptides showed (Fig. 1 *a*) that peptide 2A had lost binding ability, in accordance with the anchor character of this position, and also 9A had impaired binding, although not as much as 2A, since Ala can function as a weak anchor at position 9. Other substitutions that decreased binding were 6A and 7A, suggesting the importance of these residues as secondary anchor positions. However, peptides 4A and 8A had higher affinity, with  $\text{FI}_{0.5}$  around 2  $\mu\text{M}$ . Finally, substitutions at positions 1, 3, and 5 did not have any effect on peptide binding.

**Binding of peptides with substitutions at position 1.** C7A2-WT has aspartic acid, a negatively charged residue, in position 1, that according to previously reported data (28) has been described as a residue with a negative effect on binding to HLA-A2.1. This character made this position potentially amenable to changes to improve the binding ability. Substitution by Ala at this position, although replacing the charged residue, did not improve binding, so the original D was replaced by other residues described as having a positive effect at this position (28). Peptides with aromatic amino acids at this position (F and H), as well as N (which eliminates the negative charge but keeps the size), or S and T, small polar amino acids that can hydrogen bond, were synthesized.

As shown in Fig. 1 *b*, peptides 1N, 1H, and 1T had improved binding affinity, whereas 1F (although F has been described at this position in many HLA-A2.1 binders) had a  $\text{FI}_{0.5}$  higher than C7A2-WT, indicating lower affinity. Solubility problems with 1F may account for impaired binding ability in this case.

Table I. Sequence of the Peptides Used in the Present Study

Peptide	Sequence	Peptide	Sequence
C7A2-WT	DLMGYIPLV	1N	NLMGYIPLV
1A	ALMGYIPLV	1F	FLMGYIPLV
2A	DAMGYIPLV	1H	HLMGYIPLV
3A	DLAGYIPLV	1S	SLMGYIPLV
4A	DLMAYIPLV	1T	TLMGYIPLV
5A	DLMGAIPLV	3I	DLIGYIPLV
6A	DLMGYAPLV	3L	DLGYYIPLV
7A	DLMGYIALV	3V	DLVGYIPLV
8A	DLMGYIPAV	3K	DLKGYIPLV
9A	DLMGYIPLA	3W	DLWGYIPLV
		7G	DLMGYIGLV
		7V	DLMGYIVLV
		8V	DLMGYIPVV
		9S	DLMGYIPLS



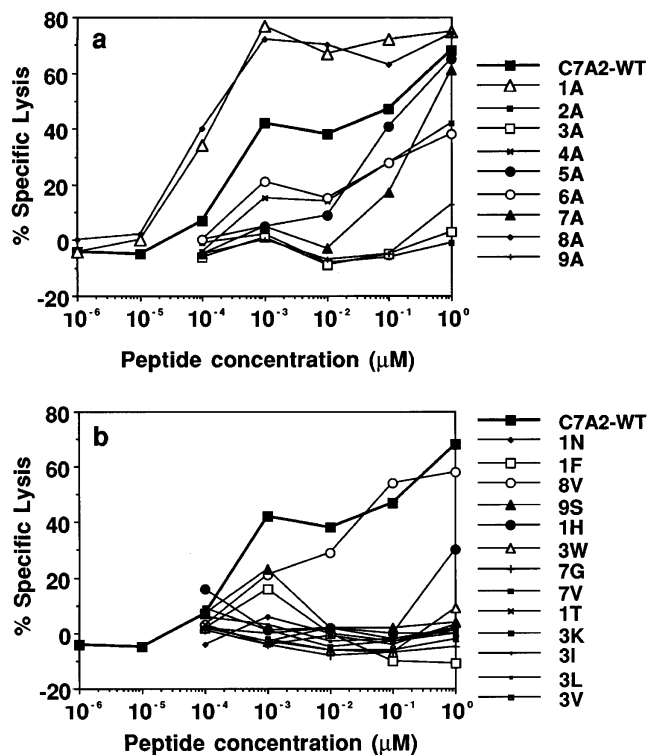
**Figure 1.** Binding of C7A2 (a) Alanine and (b) non-Alanine-substituted peptides to HLA-A2.1. T2 cells were incubated overnight in a 96-well plate in CTM with 10  $\mu\text{g}/\text{ml}$  human  $\beta 2$ -microglobulin and different peptide concentrations. Next day cells were washed, and HLA-A2.1 expression was assessed by flow cytometry using the anti-HLA-A2 BB7.2 mAb and FITC-labeled goat anti-mouse Ig. Results are expressed as fluorescence index (FI), calculated according to the formula: (mean fluorescence with peptide – mean fluorescence without peptide)/mean fluorescence without peptide. Background fluorescence without BB7.2 was subtracted for each individual value.  $\text{FI}_{0.5}$  represents those peptide concentrations that increase HLA-A2.1 expression by 50% over the no peptide control, and values were calculated from the titration curves for each particular peptide.

**Binding of peptides with substitutions at other positions.** Substitution by A at position 7 yielded a peptide with impaired binding ability. The natural amino acid at this position is P, which is known to induce turns in peptide chains. To discriminate whether the A replacement changed the structure of the peptide chain or eliminated the side chain responsible for interaction with HLA-A2.1, we synthesized 7G, an amino acid that also contributes to  $\beta$  turns as does P, but lacks the side chain, and 7V, with an aliphatic side chain bulkier than A. None of these peptides bound to HLA-A2.1 (Fig. 1 b), suggesting a specific role of the P side chain in the binding to HLA-A2.1.

Position 3 was replaced by W, because W is frequently found at this position in HLA-A2.1-binding peptides (28), and by K, L, I, and V, charged and aliphatic residues. Binding was improved with 3W (Fig. 1 b), but it was completely abolished in 3K. Peptide 3V had impaired binding, whereas 3L and 3I were similar to C7A2-WT.

Finally, it was of interest to replace position 8L with a V, an aliphatic residue similar to A and L (amino acid present in C7A2-WT), but with an intermediate size between them. Moreover, 8V is one of the few sequence variations that can be found within this epitope and is common in viral isolates belonging to HCV genotypes 2a and 2b (56, 57). Peptide 8V had an  $\text{FI}_{0.5}$  of 15  $\mu\text{M}$ , in the range of C7A2-WT.

**Recognition of C7A2-variant peptides by human CTLs.** To determine residues involved in CTL recognition, a CTL line specific for C7A2 was established from PBMCs from an HCV-infected patient after several rounds of C7A2-WT peptide stimulation. Lytic activity of these CTLs was tested with target cells incubated with different concentrations of each one of the peptides to study the recognition of the different variants and titrate the T cell avidity. In the case of Ala-substituted peptides (Fig. 2 a), 2A and 9A were not recognized, in accordance with the anchor character of these positions. Also, 3A was not recognized, despite good HLA-A2.1 binding, suggesting the epitopic character of this position. Peptides 4A, 5A, 6A, and 7A, although recognized at the highest concentrations, showed poorer recognition than C7A2-WT, as a consequence in some cases of poorer MHC binding (6A and 7A) or



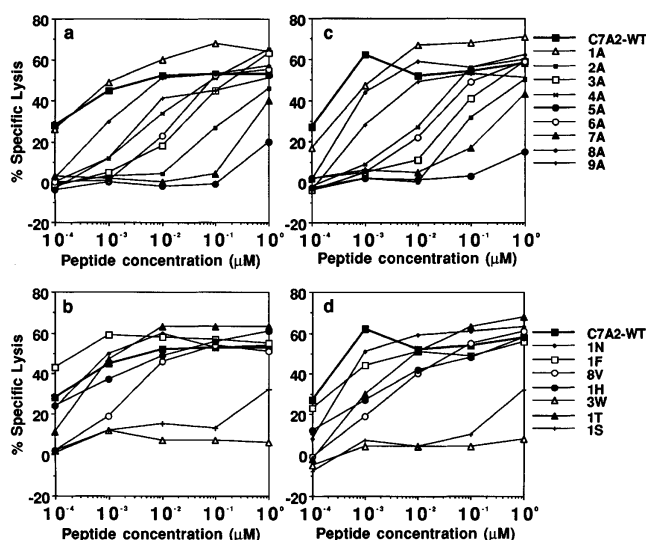
**Figure 2.** Recognition of C7A2 (a) Alanine and (b) non-Alanine-substituted peptides by human CTL line ViT2. C1R.A2.1 target cells were incubated for 2 h with  $^{51}\text{Cr}$ , washed three times, and plated at 3,000 cells/well in 96-well round-bottom plates with different peptide concentrations. After 2 h, effector cells were added (E/T ratio, 5:1), and the plates were incubated for 4 h.

otherwise of impaired T-cell recognition (4A and 5A). Finally, 1A and 8A showed higher levels of lysis than C7A2-WT, and titrated at 10-fold lower concentrations.

In the case of the non-Ala substituted peptides (Fig. 2 *b*), none of them was recognized by the CTL at the concentrations tested (including those having high binding affinity, such as 1N or 1T), with the exception of 8V, which behaved similarly to C7A2-WT, and 1H, which induced marginal lysis at the highest concentration tested.

**Recognition of C7A2-variant peptides by HLA-A2.1 transgenic mouse CTL.** AAD transgenic mice express  $\alpha 1$  and  $\alpha 2$  domains from the human HLA-A2.1 molecule and  $\alpha 3$  from the mouse D<sup>d</sup> molecule (48). Transgenic mice expressing human HLA-A2.1 molecules have been described as a model of presentation and recognition of several HLA-A2.1-restricted antigens (9, 37, 47, 58–60), and allow testing of immunogenicity in the context of a human HLA molecule prior to immunizing humans. To use our antigen in this model, recognition of the different C7A2 variants by murine CTLs *in vitro* should be studied prior to testing the immunogenicity of the different peptides *in vivo*.

CTL lines AAD.10 and AAD.1 were induced by immunization with peptide C7A2-WT together with a helper epitope presented by the H-2<sup>b</sup> class II MHC molecules of this strain and GM-CSF and IL-12 according to the method of Ahlers et al. (54). After 10–12 *in vitro* stimulations with C7A2-WT, these lines were tested against the whole panel of C7A2-substituted peptides in a lytic assay to compare the recognition of the different peptides with that by the human CTL line. In the case of the Ala-substituted peptides (Fig. 3, *a* and *c*), 1A and 8A were able to sensitize target cells in a concentration range around that of C7A2-WT, 1A being more effective than 8A for both lines. The other peptides titrated at several 10-fold concentrations higher. Peptides 5A, 7A, and 2A required the



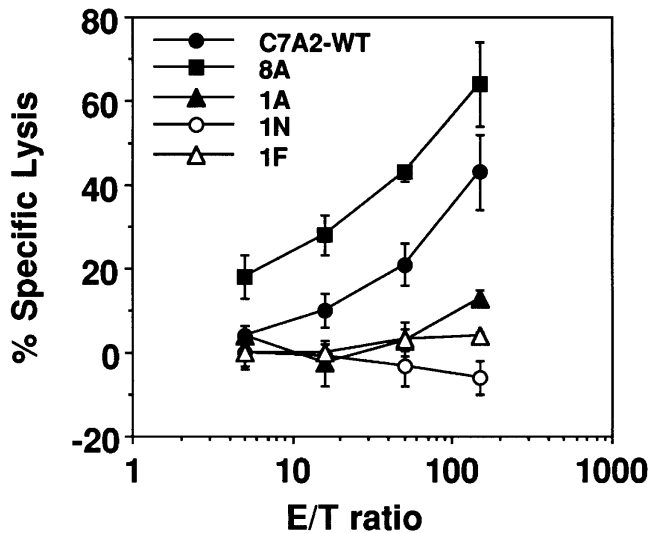
**Figure 3.** Recognition of C7A2 (*a* and *c*) Alanine and (*b* and *d*) non-Alanine-substituted peptides by transgenic AAD mouse CTL lines AAD.10 (*a* and *b*) and AAD.1 (*c* and *d*) after 10–12 *in vitro* stimulations. C1R.AAD target cells were incubated for 2 h with <sup>51</sup>Cr, washed three times, and plated at 3,000 cells/well in 96-well round-bottom plates with different peptide concentrations. After 2 h, effector cells were added (E/T ratio, 10:1), and the plates were incubated for 4 h.

highest concentrations to induce significant lysis, whereas 3A, 6A, 4A, and 9A titrated at intermediate concentrations. These results confirm that substitutions at agretopic positions as 2 and 7 decrease binding to HLA-A2.1 and almost abrogate CTL recognition. However, position 5 was clearly shown to be an epitopic residue, and almost no significant lysis was obtained with target cells sensitized with peptide 5A, as had been observed for position 3 in the human CTL line. Peptide 3A titrated at a lower concentration, suggesting that in the case of the murine HLA-A2.1-restricted CTL lines, position 3 is not involved in TCR binding as clearly as in the human line. Finally, peptides with substitutions at positions 4 and 6 were recognized as in the case of the human CTL line.

Together with Ala-substituted peptides, peptides with substitutions by other amino acids and positive binding to HLA-A2.1 were also tested in recognition by AAD mouse CTL lines (Fig. 3, *b* and *d*). These peptides mainly contained substitutions at position 1, except for peptides 8V and 3W. Surprisingly, with the exception of 1S, which required a high peptide concentration to sensitize target cells, all the peptides with substitutions at position 1 were recognized by AAD mouse CTL lines in a concentration range around C7A2-WT, and in some cases, like 1F, induced a higher response with line AAD.10. Peptide 8V was recognized at higher concentrations than C7A2-WT, whereas peptide 3W was not recognized at any of the concentrations tested.

***In vivo immunogenicity of C7A2-derived peptides in HLA-A2.1 transgenic mice.*** After studying the recognition of C7A2-derived peptides by human and AAD transgenic murine CTL, we tested the *in vivo* immunogenicity of these peptides in the AAD transgenic mouse model. Different groups of animals were immunized with the substituted CTL epitopes in conjunction with a helper epitope and cytokines as above, and their ability to induce an immune response was tested in CTL assays. In these assays were included some of the peptides with positive binding that were recognized by AAD mouse CTL lines. As shown in Fig. 4, peptides C7A2-WT and 8A were able to induce clear CTL responses, whereas 1A could induce only a marginal response. In contrast, peptides 1N and 1F were unable to induce any measurable CTL response.

***Induction of CTL immune response against C7A2-WT by C7A2-WT and 8A peptides in HLA-A2.1 transgenic mice.*** Among all the peptides tested in binding assays to HLA-A2.1 and recognition by human and transgenic mice CTL, 8A was the most promising. This peptide was able to bind at lower concentrations than C7A2-WT, sensitized human and mouse target cells in the same range or even at lower concentrations than C7A2-WT, and, in immunization experiments, induced higher levels of lysis. Therefore, the ability of 8A to induce a CTL immune response against C7A2-WT was tested in the next set of experiments. In a first experiment, each peptide was used to immunize two groups of animals, with 50 or 15 nmol of CTL epitope peptide as described in Methods. After two immunizations, spleen cells were stimulated *in vitro* with the same peptide used for immunization, and the lytic activity against C7A2-WT (and 8A in the groups immunized with 8A) was tested. As shown in Fig. 5, two of the four animals immunized with C7A2-WT were able to respond to this peptide, whereas in the animals immunized with 8A, together with positive responses to 8A itself, strong responses to C7A2-WT could be detected, even in the group of mice immunized with 15 nmol.

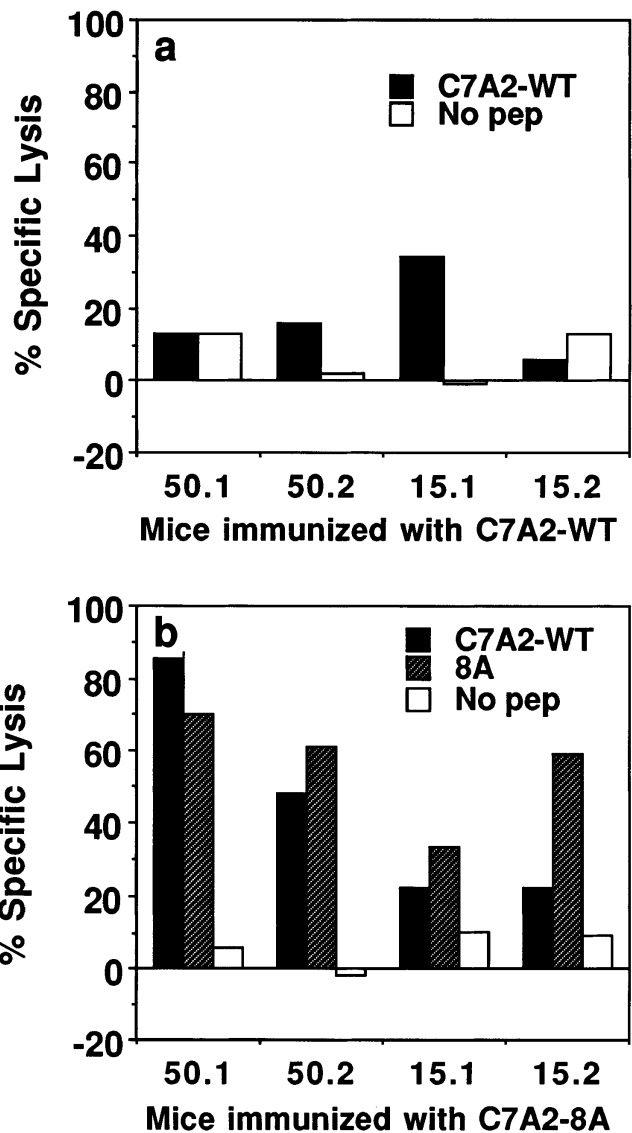


**Figure 4.** Immunogenicity of C7A2-substituted peptides in AAD transgenic mice. Mice were immunized with 50 nmol CTL epitope plus 50 nmol HBVc 128–140 helper epitope and GM-CSF and IL-12 in IFA. 2 wk later, they were boosted, and 10–14 d after the boost, spleen cells were removed and stimulated in vitro with the CTL epitope. After 1 wk of in vitro stimulation, CTL assay was performed with target cells (AAD Con A blasts) incubated with or without 10  $\mu$ M CTL peptide. CTL from each group were tested only with the peptide used to immunize those mice. Only the results for peptide-pulsed target cells are shown. In all the cases, percentages of specific lysis against peptide unpulsed target cells were below 5%.

In a second group of experiments, A2Kb transgenic mice, that express also a chimeric HLA molecule with  $\alpha 1$  and  $\alpha 2$  domains from human HLA-A2.1 and  $\alpha 3$  from mouse K<sup>b</sup>, were immunized with 50 nmol C7A2-WT or 8A, and the ability of these peptides to induce a CTL response recognizing C7A2-WT was tested. Thus, we could test immunogenicity in a different model system. Figure 6 shows that, as in the case of the AAD mice, C7A2-WT induced a low response, whereas the immunization with 8A induced a higher level of CTL that recognized both C7A2-WT and 8A, even using a lower E/T ratio in the CTL assay (70:1 vs. 120:1).

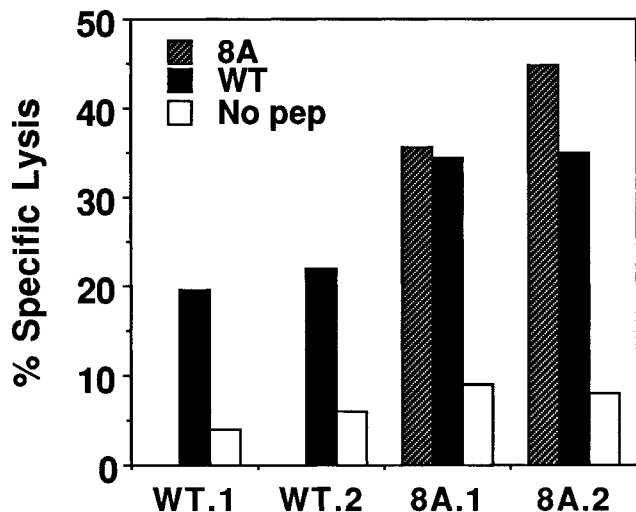
CTL avidity for their target peptide–MHC complex, defined as the sensitivity to detect low densities of peptide–MHC complex on the surface of target or stimulator cells, has been shown to make a substantial difference in the ability of CTL to clear a virus infection in vivo, in two murine model systems (61, 62). Therefore, to determine whether the CTLs raised against the modified peptide 8A were of as high avidity against targets presenting the wild-type peptide as would be CTLs raised against the wild-type peptide itself, we titrated C7A2-WT on target cells and compared the killing by short-term lines raised against this peptide or against 8A (Fig. 7). The CTLs raised against 8A killed targets expressing the C7A2-WT at concentrations more than 100-fold lower than were required to get comparable levels of killing by the CTL raised against the C7A2-WT itself. Thus, use of the modified peptide in this case increases not only immunogenicity but also the avidity of the CTLs that are produced, an additional advantage for the potential efficacy of a vaccine.

*Recognition of peptide 8V by CTLs from 8A immunized HLA-A2.1 transgenic mice.* As mentioned above, one of the



**Figure 5.** Induction of CTL immune response against C7A2-WT in AAD transgenic mice using different C7A2 peptide variants. Mice were immunized with 50 or 15 nmol CTL epitope C7A2-WT (a) or 8A (b) plus 50 nmol HBVc 128–140 helper epitope and GM-CSF and IL-12 in IFA. 2 wk later, they were boosted under the same conditions, and 10–14 d after the boost, spleen cells were removed and stimulated in vitro with 10  $\mu$ M CTL peptide-pulsed spleen cells. After 2 wk of in vitro stimulation, CTL assay was performed with target cells (AAD Con A blasts) incubated with or without 10  $\mu$ M peptide. 50.1 and 50.2, and 15.1 and 15.2 designate different mice immunized with 50 or 15 nmol CTL epitope, respectively (E/T ratio in the assay, 90:1).

peptides that is recognized by both human and mouse C7A2-WT-specific CTL lines is peptide 8V. This peptide is common in viral isolates from HCV genotypes 2a and 2b (56, 57). Since 8A has a change in the same position, it was an interesting point to know if CTLs induced by immunization with 8A were cross-reactive not only with C7A2-WT, which corresponds to viral sequences from genotypes 1a, 1b, and 3a, but also with peptide 8V, belonging to viral sequences from genotype 2. Results in Fig. 8 show that CTLs induced by immunization with 8A recognize peptide 8V both in AAD and in A2Kb mice in a way similar to the recognition of C7A2-WT.



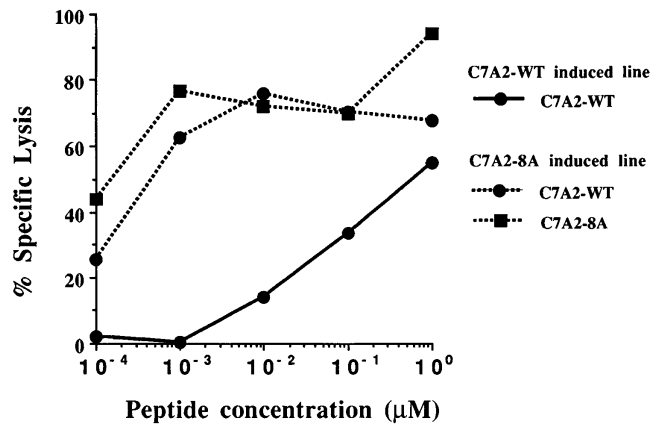
**Figure 6.** Induction of CTL immune response against C7A2-WT in A2Kb transgenic mice using different C7A2 peptide variants. Mice were immunized with 50 nmol CTL epitope C7A2-WT or 8A plus 50 nmol HBVc 128–140 helper epitope in IFA. 2 wk later, they were boosted under the same conditions, and 10–14 d after the boost, spleen cells were removed and stimulated in vitro with 10  $\mu$ M CTL peptide-pulsed spleen cells. After 2 wk of in vitro stimulation, CTL assay was performed with target cells (A2Kb Con A blasts) incubated with or without 10  $\mu$ M peptide. WT.1 and WT.2, and 8A.1 and 8A.2 designate different mice immunized with C7A2-WT and 8A CTL epitope, respectively (E/T ratio in the assay, 120:1 for WT.1 and WT.2, 70:1 for 8A.1 and 8A.2).

## Discussion

A CTL response in HCV infection is found in PBMCs (8–14) and in the liver of patients and infected chimpanzees (15, 16), but despite the importance of CTLs in the clearance of virus in many infections, in the case of HCV, the CTL response does not seem to be adequate to achieve complete recovery (17). Improvement of epitopes recognized in the natural infection might lead to an enhancement of the antiviral immune response, and ultimately, together with other effector arms of the immune system, might help in viral clearance.

For several years, our lab has focused on an “epitope enhancement” approach to making vaccines that can be more effective than the natural virus at inducing T cell responses (38, 42–45, 63). By modifying the sequence of an epitope to increase affinity for the MHC molecule without altering what the T cell sees, one can potentially make a more potent immunogen capable of inducing T cells specific for the natural viral sequence. Most of our experience so far is with helper T cells and binding to class II MHC molecules, but we hypothesize that the same approach could be applied to class I MHC molecules presenting to CTLs. Indeed, a few examples of improved binding to class I MHC molecules have been reported (34, 35).

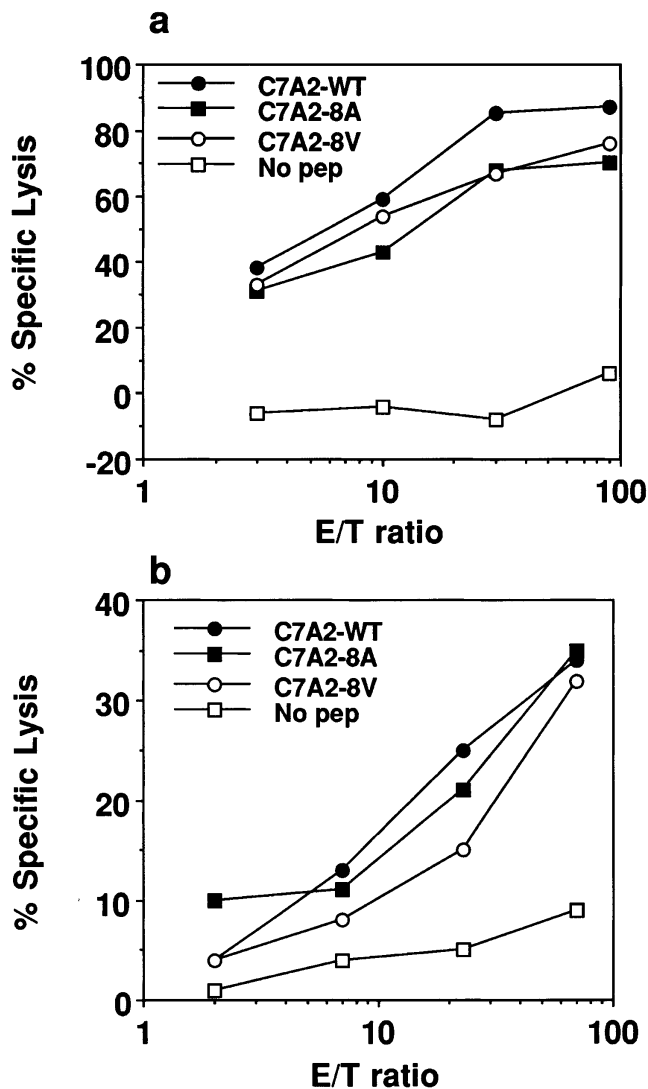
In this report, the CTL response against modified peptides from a HCV HLA-A2.1 restricted epitope has been analyzed. This epitope has an intermediate HLA binding affinity (12, 30), is recognized by CTLs from HCV-infected patients (9, 10, 12), and induces CTL responses in transgenic mice bearing HLA-A2.1 molecules (9, 37). In a first approach, HLA-A2.1 binding studies with Ala-substituted peptides showed the role of each residue. Thus, as expected by the presence of L at posi-



**Figure 7.** Avidity of short-term CTL lines induced with C7A2-WT and C7A2-8A. AAD transgenic mice were immunized as described in the Methods with 50 nmol CTL epitope C7A2-WT (solid lines) or C7A2-8A (dotted lines) plus 50 nmol HBVc 128–140 helper epitope and GM-CSF and IL-12 in IFA. 2 wk later, they were boosted under the same conditions, and 10–14 d after the boost, spleen cells were removed and stimulated in vitro with 10  $\mu$ M CTL peptide-pulsed spleen cells. After three to four in vitro stimulation cycles, the CTL assay was performed with target cells (C1R.AAD) incubated with different peptide concentrations as indicated (circles, targets with C7A2-WT peptide; squares, targets with C7A2-8A peptide), at an E/T ratio of 10:1.

tion 2 and V at position 9, these amino acids behaved as anchor residues (22, 23). Moreover, positions 6 and 7 were also involved in binding, because Ala substitution at these positions almost abrogated binding to HLA-A2.1, and more surprisingly, replacement of position 4 and 8 by Ala yielded peptides with a higher binding affinity. Substitutions of D at position 1 by aromatic and polar residues resulted also in peptides with a higher affinity for HLA-A2.1, in accordance with previous reports (28, 29). Some of these peptides with an improvement in their sequence were of great interest in testing in CTL assays, in order to study whether these replacements had increased MHC class I binding without impairing CTL activity.

Substituted peptides were tested in CTL assays, and 1A and 8A showed higher CTL activity than C7A2-WT when tested with a C7A2-WT-specific human CTL line, and both 1A and 8A, together with other peptides with substitutions at position 1, were recognized by C7A2-WT-specific CTL lines raised in HLA-A2.1 AAD transgenic mice. Although all the lines recognized the same epitope, differences in fine specificity between lines may be explained by differences in the TCR specificity. Thus, position 3 is the main epitopic residue in the human line (substitution 3A completely abolishes CTL recognition without affecting HLA-A2.1 binding), whereas position 5 is the key epitopic residue in the mouse lines. A possible explanation for the differences in fine specificity between these lines would be that the TCR in the human line contacts the peptide around the NH<sub>2</sub>-terminal region residues, and therefore, changes at position 1, although they improve peptide binding, may result in conformational changes that affect the peptide–MHC structure recognized by the TCR around position 3. However, mouse TCRs recognized C7A2 around position 5, and replacements at position 1 would improve binding and at the same time did not affect peptide structure for CTL recognition. As a result of these experiments, substitutions at



**Figure 8.** Recognition of C7A2-derived peptides from different HCV genotypes by CTL induced following immunization with peptide 8A. (a) AAD and (b) A2Kb transgenic mice were immunized with 50 nmol CTL epitope 8A plus 50 nmol HBVc 128–140 helper epitope in IFA. 2 wk later, they were boosted under the same conditions, and 10–14 d after the boost, spleen cells were removed and stimulated in vitro with 10  $\mu$ M CTL peptide-pulsed spleen cells. After 2 wk of in vitro stimulation, CTL assay was performed with target cells (Con A blasts) incubated with the different peptides at 10  $\mu$ M. C7A2-WT has a sequence from HCV genotypes 1a, 1b, and 3a, whereas peptide C7A2-8V has a sequence from genotypes 2a and 2b.

agretopic residues were more attractive in order to enhance CTL activity, because replacement at these positions would probably not affect interactions between the TCR and the peptide–MHC complex, whereas replacement of epitopic residues might have different effects depending on the characteristic specificity of each particular line.

To use the epitopes as components of a vaccine aimed at enhancing anti-HCV immune response, the activity as in vivo immunogens of some of the peptides previously tested was studied in the AAD HLA-A2.1 transgenic mouse model. This model allows one to test immunogenicity in vivo of peptides presented by human HLA molecules without having to immu-

nize humans, and the response in the transgenic mice has been shown to be predictive of that in humans (9, 49). Of all the peptides tested, only 8A showed a higher immunogenicity than C7A2, whereas peptides with substitutions at position 1 were unable to induce any significant response, although they were recognized by C7A2-specific CTL lines. Together with this high in vivo immunogenicity, recognition of C7A2 by 8A-specific CTLs was tested, showing that CTL responses against C7A2 were stronger after immunization with 8A than the responses induced by C7A2 itself in both HLA-A2.1 AAD and A2Kb transgenic mice strains. These results are in agreement with several reports showing a relationship between MHC class I binding and in vitro (30, 36) and in vivo (30, 32, 33, 58) immunogenicity. In all these cases, a high MHC class I binding affinity correlated with the ability to induce a response in immunized animals or to stimulate in vitro cells from virus-infected or cancer patients. We suggest that increased affinity for MHC can enhance in vivo immunogenicity by increasing the density of peptide–MHC complexes on antigen-presenting cells, a property shown to be important for the induction of a CTL response (64).

Another important property of CTL to be induced by a vaccine is high avidity for the target antigen, that is ability to respond to low densities of peptide–MHC complex on the target cell, as we (61) and others (62) have shown that this property is essential for optimal clearance of virus infection in vivo. Therefore, we tested whether the CTL raised by immunization with the 8A peptide killed targets presenting the C7A2-WT peptide at concentrations as low as or lower than those required for killing by CTL raised by immunization with the C7A2-WT peptide itself. Indeed, we found that the CTL raised against 8A had substantially higher avidity for the complex of HLA-A2.1 and the C7A2-WT peptide than did the CTL raised against the wild-type peptide. Thus, these CTLs may be qualitatively better than those raised against the wild-type peptide, in addition to being quantitatively greater in numbers. We conclude that the modified peptide 8A has at least two advantages as a vaccine, being more immunogenic and inducing higher avidity CTL. Both of these properties should contribute to enhanced efficacy in a vaccine.

Nevertheless, empirical characterization of in vivo immunogenicity of the peptides to be used as immunogens is important, since we found in this study that good MHC class I binding affinity and CTL recognition are not always sufficient to induce a CTL response. Among all the peptides tested, only 8A was a good immunogen. The reasons the other peptides were unable to stimulate a primary immune response remain unknown. The most interesting example in this last group is 1A, a peptide with good recognition by both human and mouse CTL but a very poor in vivo immunogen. These results point out the importance of testing the ability of the peptides to induce a good in vivo immune response before they are considered to be included in a vaccine. In our case, the HLA-A2.1 transgenic mouse model has been a valuable system to study this property of the different peptides. Although there may be some differences in fine specificity between human and mouse T cells, substitutions at positions involved in MHC class I binding can avoid affecting TCR recognition, so any change in positions modifying HLA-A2.1 binding are likely to have the same effect both in mouse and human CTL activity.

Together with the higher immunogenicity, recognition of peptides belonging to other viral genotypes by peptide 8A–



induced CTL was another point of importance to vaccine development. HCV is a virus with a high degree of variation, and several types and subtypes have been defined in different geographical areas (65). C7A2 is an epitope located in a conserved region within the HCV genome, and the sequence used for the present study is common in viral isolates from genotypes 1a, 1b, and 3a (1, 66–68). Among all the peptides tested, 8V had the sequence of viral isolates from genotypes 2a and 2b (56, 57), and despite having a mutation at position 8 (as peptide 8A), 8A-induced CTL recognized 8V-pulsed target cells similarly to cells pulsed with C7A2. This recognition may be explained because substitution by V at position 8 still retains an aliphatic side chain, with an intermediate size between that of L and A, allowing binding to HLA-A2.1 without affecting interactions with the TCR. Recognition of 8V was also seen in the case of C7A2-induced CTL. Thus, together with the enhancement of immunogenicity, broad recognition of viral genotype sequences was obtained with 8A-induced CTL.

Enhanced immunogenicity and induction of an immune response with a broad specificity for different viral genotypes make 8A a good candidate to be included as a component in a subunit vaccine. It can be included and delivered in a peptide vaccine, as described for HBV infection (69), in a recombinant virus, or in a nucleic acid vaccine with HCV sequences containing the replacements made in the appropriate subunits. This approach of modifying epitopes to enhance their immunogenicity has been successfully used in different systems, with viral (34) or tumor (35) antigens. This is the first case, to our knowledge, where the improvement of a viral CTL epitope recognized by human cells has been tested both in vitro and in vivo. This epitope enhancement approach could be used also with CD4 T-helper epitopes, as described in other systems (38, 45), and antigens carrying combinations of improved epitopes recognized by different lymphocyte populations could be incorporated in a vaccine designed to enhance the antiviral immune response. Moreover, we have shown peptide 8A to be more potent than C7A2 in stimulating in vitro the CTL response induced in vivo by viral infection, as in the case of the human CTL line, suggesting that it could also be used in immunotherapy to enhance already existing CTL activity.

In conclusion, replacement of secondary anchor residues in a HLA-A2.1-restricted CTL epitope from a conserved region of the HCV core protein yielded a peptide with enhanced in vitro and in vivo immunogenicity, as tested in human and HLA-A2.1 transgenic mice models. This peptide also induces CTL recognizing sequences from a broad spectrum of viral genotypes. These results suggest that this peptide could be used to induce CTL in vitro for adoptive transfer immunotherapy and as a component in a vaccine aimed at clearing HCV infection. Moreover, the results demonstrate proof of principle of this epitope enhancement strategy, which could also be used for other epitopes in HCV antigens to enhance antiviral immune response.

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