

# Molecular Mimicry of Human Cytochrome P450 by Hepatitis C Virus at the Level of Cytotoxic T Cell Recognition

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## Summary

Hepatitis C virus (HCV) is thought to be involved in the pathogenesis of autoimmune hepatitis (AIH) type 2, which is defined by the presence of type I antiliver kidney microsome autoantibodies directed mainly against cytochrome P450 (CYP)2D6 and by autoreactive liver infiltrating T cells. Virus-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) that recognize infected cells and contribute to viral clearance and tissue injury during HCV infection could be involved in the induction of AIH. To explore whether the antiviral cellular immunity may turn against self-antigens, we characterized the primary CTL response against an HLA-A\*0201-restricted HCV-derived epitope, i.e., HCV core 178–187, which shows sequence homology with human CYP2A6 and CYP2A7 8–17. To determine the relevance of these homologies for the pathogenesis of HCV-associated AIH, we used synthetic peptides to induce primary CTL responses in peripheral blood mononuclear cells of healthy blood donors and patients with chronic HCV infection. We found that the naive CTL repertoire of both groups contains cross-reactive CTLs inducible by the HCV peptide recognizing both CYP2A6 and CYP2A7 peptides as well as endogenously processed CYP2A6 protein. Importantly, we failed to induce CTLs with the CYP-derived peptides that showed a lower capacity to form stable complexes with the HLA-A2 molecule. These findings demonstrate the potential of HCV to induce autoreactive CD8<sup>+</sup> CTLs by molecular mimicry, possibly contributing to virus-associated autoimmunity.

**Key words:** hepatitis C-like viruses • autoimmunity • hepatitis, autoimmune • HLA-A2 antigen • antigens, viral

**A**utoimmune hepatitis (AIH)<sup>1</sup> is a chronic liver disease of unknown etiology characterized by a persistent inflammatory reaction in the liver. The hallmarks of AIH are high titers of serum autoantibodies against different autoantigens and the presence of a hepatic, predominantly mononuclear cell infiltrate associated with liver cell damage. Several reports have shown the presence of autoreactive T cells in AIH patients. CD4<sup>+</sup> or CD8<sup>+</sup> liver-infiltrating T cells proliferate in response to autologous hepatocytes and some of them

express high cytolytic activity (1). Peripheral T cell clones from AIH patients also proliferate in response to liver-specific lipoprotein and the asialoglycoprotein receptor (2). Type 2 AIH is defined by the presence of type I antiliver kidney microsome antibodies (LKM-1) recognizing cytochrome P450 (CYP)2D6 (3). In the liver and blood of patients with AIH type 2, CD4<sup>+</sup> and CD8<sup>+</sup> autoreactive T cells recognizing CYP2D6 have been detected, indicating a role of T cells in the pathogenesis of this disease (4, 5). AIH type 2 appears to be epidemiologically linked to hepatitis C virus (HCV) infection. Some patients with AIH type 2 have been shown to be positive for anti-HCV antibodies (6) and also for HCV RNA (7), suggesting that HCV may cause a secondary autoimmune response. MHC class I-restricted CD8<sup>+</sup> CTLs are a major defense mechanism in viral infections. It has been suggested that the CTL response may

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<sup>1</sup>Abbreviations used in this paper: AIH, autoimmune hepatitis; APL, altered peptide ligand; CYP, cytochrome P450; HCV, hepatitis C virus; LKM-1, type I antiliver kidney microsome antibodies; MF, mean fluorescence; PBA1%, PBS containing 1% BSA.

contribute to viral clearance as well as to liver cell injury during HCV infection and might therefore play a role in the induction of autoreactive antibodies and CD4<sup>+</sup> helper T cells. CTLs recognize endogenously processed antigenic peptides in combination with MHC class I molecules presented on the cell surface. Several HCV-derived immunogenic CTL epitopes have been described thus far (8–13). Amino acid sequence comparison of HLA-A2–restricted HCV-derived CTL epitopes revealed the presence of two human CYP sequences related to HCV core 178–187, i.e., CYP2A6 8–17 and CYP2A7 8–17 (Table I). Compared with the HCV core 178 epitope, they display an eight amino acid sequence identity, one conservative substitution at the NH<sub>2</sub>-terminal anchor position (Leu to Val), and one non-conservative substitution at position 6 (Ser to Val or Ser to Ala). Both CYP sequences still contain the HLA-A2 binding motif, because both Leu and Val can serve as anchor residues (14, 15). Therefore, the HCV core 178 peptide is a candidate for molecular mimicry, that is, similarity of infectious agents with host antigens. Such similarity may lead to an inability of the host immune system to recognize the foreign antigen or it may lead to an autoreactive immune response at the level of antibodies or T cells (16–18).

Molecular mimicry at the level of CD4<sup>+</sup> T cells seems to play a role in several human autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, myocarditis, and herpes stromal keratitis (19–23). A report describing a possible molecular mimicry at the level of CD8<sup>+</sup> T cells has also been published (24).

To determine the relevance of the sequence similarities between an HCV-derived CTL epitope and members of the CYP family for pathogenesis of HCV-associated autoimmune hepatitis, we analyzed the primary CTL repertoire, inducing primary CTL responses in the PBMCs of HCV-negative, healthy blood donors as well as those of patients with chronic HCV infection without markers for AIH. For induction, we used the HCV core 178 epitope as well as both CYP-derived peptides. CTL were then tested for their ability to recognize all three different peptide ligands.

## Materials and Methods

**Study Population.** 12 HLA-A2–positive healthy individuals and 10 HLA-A2–positive patients with chronic hepatitis C were

**Table I.** HCV Core 178–187 and Related Human Cytochrome P450 Amino Acid Sequences

Origin	Residues	Sequence*
HCV-1 core	178–187	LLALLSCLTV
Cytochrome P450 2A6	8–17	·V···V···
Cytochrome P450 2A7	8–17	·V···A···

\*Amino acid sequence comparison was performed using the SwissProt data base and identified two related human cytochrome P450 sequences. Identical amino acids are shown as dots.

studied. Samples of patients with chronic HCV infection were obtained at least 6 mo after diagnosis of HCV infection. All patients repeatedly tested positive for anti-HCV antibodies, and with one exception HCV RNA was detected by PCR. Most (8 out of 10) of the patients had not been treated with IFN- $\alpha$  before sample collection, and the other two patients finished an ineffective IFN- $\alpha$  treatment before sample collection. None of the HCV patients tested positive for autoimmune hepatitis serum markers. Patients and healthy blood donors were negative for antibodies to HIV and HBV and healthy donors were also negative for anti-HCV antibodies.

**Cell Lines.** The EBV-transformed B cell line JY (HLA-A\*0201, -B7, -Cw7, -DR4, -Drw6, and -Dpw2) and K562 cells were cultured in RPMI 1640 medium supplemented with 1-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50  $\mu$ g/ml), and Hepes (5 mM) containing 10% (vol/vol) heat-inactivated FCS (FCS-medium). The human B lymphoblastoid cell line AHH-1 TK<sup>+/–</sup> derived from the RPMI 1788 cell line (HLA type: A2, Aw33, B7, B14) was maintained in FCS-medium. H2A3 and h2D6 cells (AHH-1 TK<sup>+/–</sup> cells transfected with vectors coding for human CYP2A6 and CYP2D6, respectively; reference 25) were cultured in selective RPMI 1640 medium with 2 mM 1-histidinol without 1-histidine (Gentest Corp.) supplemented with 1-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50  $\mu$ g/ml), and Hepes (5 mM) containing 10% (vol/vol) heat-inactivated FCS (h2-medium). AHH-1 T/K<sup>+/–</sup>, h2A3, and h2D6 cells were a gift from Charles L. Crespi (Gentest Corp., Woburn, MA).

**Induction of Primary CTLs.** PBMCs from HLA-A2–positive healthy donors and HCV patients were isolated on Ficoll-Paque density gradients and washed three times in PBS containing 10% FCS-medium.  $4 \times 10^6$  PBMCs were incubated with synthetic peptide (10  $\mu$ g/ml; Chiron Mimotopes) in RPMI 1640 medium supplemented with 1-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50  $\mu$ g/ml), and Hepes (5 mM) containing 10% (vol/vol) heat-inactivated human AB serum (AB-medium) for 1 h, washed once in PBS containing 10% FCS-medium, and then plated in 24-well plates at  $4 \times 10^6$  cells/well in AB-medium. On day 3 and weekly thereafter, 1 ml of complete medium supplemented with rIL-2 (20 U/ml; EuroCetus B.V.) was added to each well. On day 7 and weekly thereafter, the cultures were restimulated with  $10^6$  peptide-pulsed, irradiated (7,400 rads) autologous feeder cells in 1 ml of AB-medium containing rIL-2 (20 U/ml). The cultured PBMCs were tested for CTL activity against different peptides on day 35.

**Generation of Peptide-specific Cell Lines.** Peptide-specific induction cultures were depleted of CD4<sup>+</sup> cells using negative selection according to the manufacturer's instructions (Dynabeads<sup>®</sup>; DYNAL A.S.) and plated at 100 cells per well in 96-well plates. Cells were plated in FCS-medium in the presence of PHA (1  $\mu$ g/ml), rIL-2 (30 U/ml), irradiated (10,400 rads) allogeneic PBMCs ( $10^6$  cells/ml), and irradiated (22,000 rads), peptide-pulsed (10  $\mu$ g/ml; 1 h) JY EBV-B cells ( $10^5$  cells/ml). Peptide-specific wells were expanded by restimulation in a 24-well plate as described above.

**Cytotoxicity Assay.** JY target cells were incubated with synthetic peptides (10  $\mu$ g/ml) in FCS-medium overnight. Target cells (peptide-pulsed JY cells or AHH-1 TK<sup>+/–</sup>-derived cells) were labeled with 100  $\mu$ Ci of Na<sub>2</sub>[<sup>51</sup>Cr]O<sub>4</sub> (Amersham Pharmacia Biotech) for 1 h and washed four times with PBS containing 10% FCS-medium. Cytolytic activity was determined in a standard 4-h <sup>51</sup>Cr-release assay using U-bottomed 96-well plates containing 2,500 targets per well. Where indicated in the figure legends, 2,500 K562 cells per well were added to reduce unspecific lysis. Percentage of cytotoxicity was determined from the

formula:  $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$ . Maximum release was determined by lysis of targets with HCl. Spontaneous release was <25% of maximal release in all assays. Specific lysis was calculated as difference between lysis of targets with peptide (or plasmid) and targets without peptide (or plasmid).

In peptide titration experiments, JY target cells were incubated with various peptide concentrations for 90 min after  $^{51}\text{Cr}$  labeling, washed once with PBS, and used as described above. In functional MHC binding assays, JY target cells were pulsed with synthetic peptide for 90 min at indicated times before the assay, then washed twice with PBS containing 10% FCS-medium, incubated in FCS-medium until  $^{51}\text{Cr}$  labeling, and used as described above. CD8 dependency of target recognition was tested by addition of 10  $\mu\text{g}/\text{ml}$  anti-CD8 antibody OKT8 (Ortho Diagnostic Systems Inc.) during the cytotoxicity assay.

**Peptide "Stripping" by Mild Acid Treatment.** JY cells were washed twice with PBS and then put on ice for 5 min.  $10^7$  cells were then treated for 90 s with 2 ml ice-cold citric acid- $\text{Na}_2\text{HPO}_4$  buffer (a mixture of an equal volume of 0.263 M citric acid and 0.123 M  $\text{Na}_2\text{HPO}_4$ ), pH 3.2. Immediately thereafter, the eluted cells were buffered with cold IMDM, washed with IMDM, and resuspended at  $5 \times 10^5$  cells in IMDM with 1  $\mu\text{g}/\text{ml}$   $\beta_2$ -microglobulin (Sigma Chemical Co.).

**Competition-based MHC Class I Peptide Binding Assay.** Peptides were tested for their binding affinity using the previously described peptide binding assay (26). In brief, cells were stripped (see above) and resuspended at  $7 \times 10^5$  cells/ml in IMDM plus 1.5  $\mu\text{g}/\text{ml}$   $\beta_2$ -microglobulin. A fluorescein (FL)-labeled reference peptide (FLPSDC(FL) FPSV), 25  $\mu\text{l}$  (end concentration, 150 nM), was incubated with 25  $\mu\text{l}$  of competitor peptide (different end concentrations) in a 96-well V-bottomed plate. 100  $\mu\text{l}$  of mild acid-treated JY cells was added to these wells. The mixture was incubated for 24 h at 4°C, washed twice with PBS containing 1% BSA (PBA1%), resuspended in PBA1% containing 0.5% paraformaldehyde, and analyzed by FACScan® (Becton Dickinson).

The mean fluorescence (MF) value obtained in the wells without competitor peptide was regarded as maximal binding and equated to 0% inhibition; the MF obtained from the wells without reference peptide was equated to 100% inhibition. Percentage of inhibition of binding was calculated using the formula:  $[1 - (\text{MF } 150 \text{ nM reference and competitor peptide} - \text{MF no reference peptide}) / (\text{MF } 150 \text{ nM reference peptide} - \text{MF no reference peptide})] \times 100\%$ .

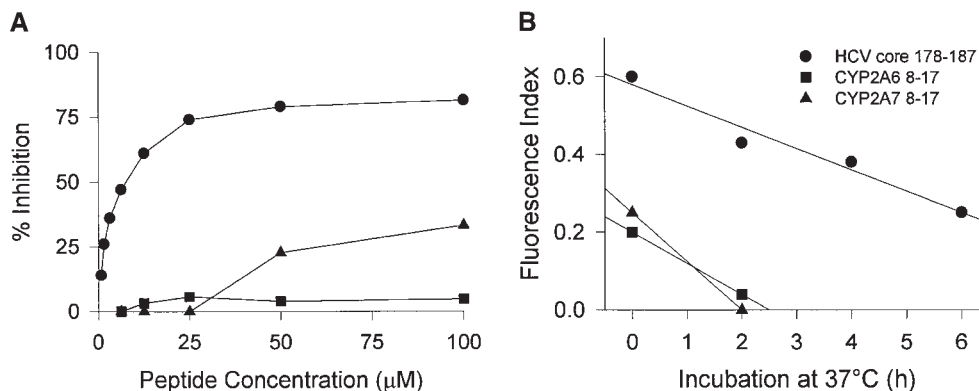
**Measurement of MHC–Peptide Complex Stability.** JY cells at a concentration of  $1\text{--}2 \times 10^6$  cells/ml were incubated with  $10^{-4}$  M emetine (Sigma Chemical Co.) for 1 h at 37°C to stop protein synthesis and the subsequent emergence of de novo synthesized

class I molecules at the cell surface. Cells were washed twice with PBS and peptide stripped (see above).  $10^6$  cells were added to 200  $\mu\text{g}$  of peptide in 1 ml and incubated for 90 min at room temperature. Cells were washed twice with ice-cold IMDM and resuspended in 1 ml IMDM. Subsequently, the cells were incubated for 0, 2, 4, and 6 h at 37°C and thereafter stained with BB7.2, an HLA-A2 confirmation-specific mAb (27), and goat anti-mouse FITC. Thereafter, the cells were fixed by resuspension in PBA1% containing 0.5% paraformaldehyde and analyzed by FACScan®. The fluorescence index (FI) was calculated as  $\text{FI} = (\text{mean fluorescence sample} - \text{mean fluorescence background}) / \text{mean fluorescence background without peptide}$ . Samples were tested in duplicate and the variation between both samples was always <10%.

## Results

**HLA-A2 Binding Affinity and HLA-A2–Peptide Complex Stability.** To characterize the HLA-A2 binding properties of HCV core 178 and the homologous CYP peptides, we determined their affinity to HLA-A2 and the stability of the formed HLA-A2–peptide complexes. The HCV core 178 bound to HLA-A2 with intermediate affinity as previously described (28), whereas both CYP peptides bound with low affinity (Fig. 1 A). Similar results were obtained measuring the peptide-induced stabilization of HLA-A2 molecules at the surface of transporter-associated with antigen processing-deficient T2 cells (data not shown). Determination of MHC–peptide complex stability showed that the HCV core 178 peptide was able to form stable complexes with a half-life of  $\sim 5$  h. The CYP2A6 8–17 as well as the CYP2A7 8–17 peptides dissociated much faster with half-lives of  $\sim 1$  h (Fig. 1 B).

**Induction of Primary CTLs.** To determine the effect of the different HLA-A2 binding properties on T cell activation and antigen recognition by CTLs, we first analyzed the naive CTL repertoire in healthy blood donors. PBMCs from 12 healthy HCV-seronegative, HLA-A2–positive blood donors were stimulated with synthetic HCV core 178–187, CYP2A6 8–17, or CYP2A7 8–17 peptide in four replica cultures. After 5 wk, the cultures were tested for CTL activity against target cells presenting each of the three peptides. Long-term stimulation with the HCV core 178 peptide induced HCV core 178-specific CTLs in nine HCV-seronegative blood donors (Table II). In five individuals, the



**Figure 1.** HLA-A2 binding affinities and dissociation rates of HCV core 178–187, CYP2A6 8–17, and CYP2A7 8–17 peptides. HLA-A2 binding affinities (A) and HLA-A2–peptide complex stabilities (B) of HCV core 178 (●), CYP2A6 8 (■), and CYP2A7 8 (▲) were determined as described in Materials and Methods. In B, the lines represent linear regression.

**Table II.** Phenotype Distribution of HCV Core 178-inducible CTLs in Healthy Donors and Patients with Chronic HCV Infection

Peptides recognized	Healthy donors* (n = 12)	Chronic HCV patients* (n = 10)
HCV core + CYP2A7		
+ CYP2A6	2 (17%)	2 (20%) <sup>‡</sup>
HCV core + CYP2A7	3 (25%)	2 (20%)
HCV core	4 (33%)	4 (40%)
None	3 (25%)	2 (20%)

\*Number (percentage) of individuals recognizing the indicated peptides after primary induction with HCV core 178. CTL responses of >15% specific lysis at an E/T ratio of 40:1 in primary induction cultures were considered positive.

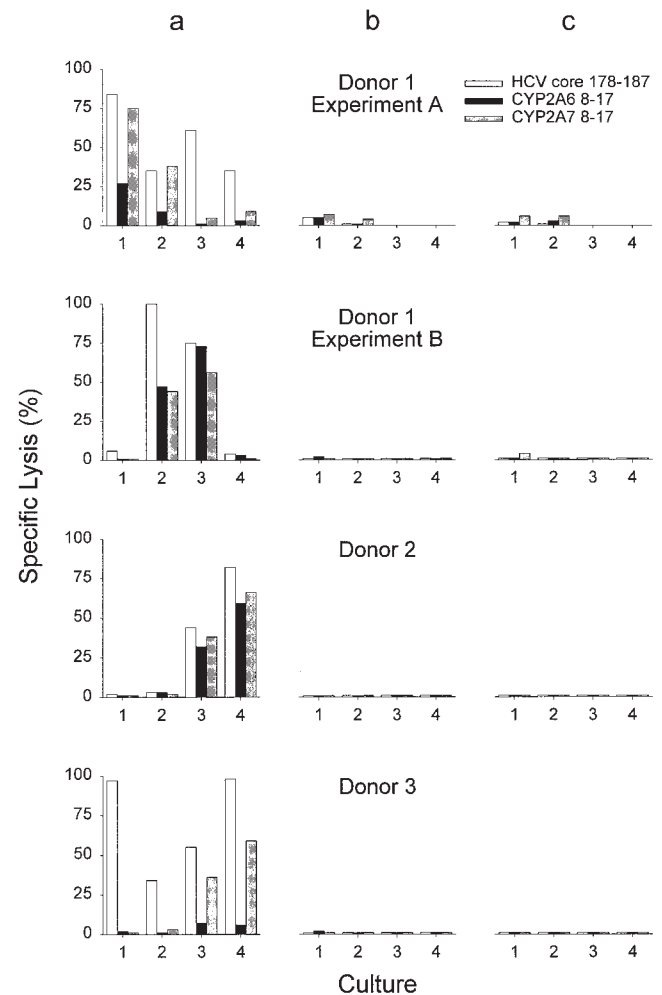
<sup>‡</sup>One of these patients was not positive for CYP2A6 according to the criteria mentioned above, but CYP2A6-recognizing cell lines could be established by limiting dilution.

HCV core 178-specific CTLs not only recognized the inducing HCV peptide but also CYP2A6 and/or CYP2A7 self-peptides. A higher specific lysis of targets presenting CYP2A7 was observed in most cases. Two donors recognized all three peptides, three individuals recognized HCV core 178 and CYP2A7 8–17, four recognized HCV core 178 only, and three did not show a CTL response after stimulation with HCV core 178. Representative results of three donors are shown in Fig. 2 a. These results as well as data obtained from CTL lines derived from positive cultures (data not shown) indicate the presence of three phenotypes of HCV core 178-specific CTLs: CTLs recognizing HCV core 178 only, CTLs cross-reactive with HCV core 178 and CYP2A7, and CTLs specific for HCV core 178, CYP2A7, and CYP2A6. We did not find CTLs recognizing CYP2A6 without recognition of CYP2A7, nor cells recognizing one of the CYP peptides without recognition of HCV core 178. Importantly, no CTL response could be induced with the self-peptides CYP2A6 8–17 (Fig. 2 b) and CYP2A7 8–17 (Fig. 2 c) in any of the 12 donors tested. This fits with our observation of different MHC-peptide interactions, because peptide-induced MHC stability and immunogenicity of the peptide are strongly correlated (28).

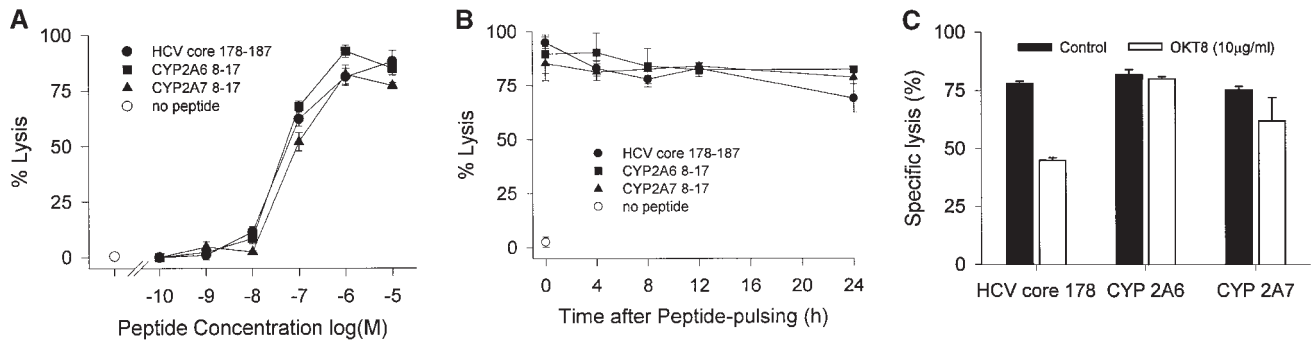
The same strategy was used to analyze the HCV core 178-specific CTL repertoire in patients with chronic HCV infection without markers for AIH. The percentage of patients having cross-reactive CTLs is comparable to that of healthy donors (Table II), suggesting that the peripheral pool of naive CTLs specific for HCV core 178 is comparable to the one of uninfected individuals and that the precursor frequency of cross-reactive CTLs is similar. This is not astonishing, as chronic HCV infection is associated with a low number of CTLs in the peripheral blood (29, 30).

**Peptide Recognition by Cross-reactive CTL Lines.** HCV core 178-induced CTL lines derived from donor 2 were used to test the biologic function of the peptides in cytotoxicity assays. Despite different MHC binding affinities and abilities

to stabilize MHC complexes, all three peptides were recognized by CTLs with the same efficiency (Fig. 3 A). The higher off-rate of the CYP peptides had no effect on CTL recognition when the peptide-pulsed target cells were further incubated without peptide for up to 24 h before exposure to CTLs (Fig. 3 B). An explanation for this phenomenon could be the CD8 dependency of target recognition. Although lysis of target cells presenting HCV core 178 is markedly reduced by the anti-CD8 antibody OKT8, recognition of CYP2A7 is less affected and there is no effect on recognition of CYP2A6 (Fig. 3 C). These findings were unexpected but similar findings had been observed by al-Ramadi et al., demonstrating that the pattern of functional activities of variant peptides does not always correlate with MHC binding (31). In fact, other mechanisms involved in the interaction between the CTL and the target cell may be important, such as the TCR affinity for the



**Figure 2.** Peptide-specific cytotoxicity induced in vitro with HCV core 178–187, CYP2A6 8–17, or CYP2A7 8–17 peptides. PBMCs from healthy HCV-seronegative blood donors were stimulated with HCV core 178–187 (a), CYP2A6 8–17 (b), or CYP2A7 8–17 (c) in four replicas (1–4). After 5 wk of stimulation, peptide-specific lysis of each culture was determined on JY target cells pulsed with HCV core 178–187 (white bars), CYP2A6 8–17 (black bars), or CYP2A7 8–17 (gray bars). E/T cell ratios were fixed for each culture and ranged from 20:1 to 40:1 for the different cultures.



**Figure 3.** Peptide recognition by CTL lines. Cross-reactive CTL lines derived from donor 2 were tested for their ability to lyse (A) JY target cells preincubated at various concentrations of peptide and (B) JY target cells pulsed with 10  $\mu$ M peptide and further incubated without peptide before exposure to CTL. Peptides were HCV core 178–187 (●), CYP2A6 8–17 (■), and CYP2A7 8–17 (▲). (C) The effect of anti-CD8 antibodies on target cell recognition was tested by addition of OKT8 antibody (10  $\mu$ g/ml) during the cytotoxicity assay. Shown are the mean  $\pm$  SD of three to four replicas at an E/T ratio of 10:1.

MHC–peptide-complex, and CD8 binding to MHC class I, as well as other costimulatory and cell adhesion molecules (32). To assess the role of CD8 binding on CTL–target cell interaction, we used a panel of three different anti-CD8 antibodies and tested their effect on target cell lysis. A representative experiment is shown in Fig. 3 C using the OKT8 antibody. Although recognition of HCV core 178 depends in part on CD8 availability, self-peptide recognition of CYP2A6 does not. Further studies will be required to define the mechanism responsible for the discrepancy observed between peptide–MHC binding and CTL-mediated cytotoxicity.

**Recognition of Endogenously Synthesized Antigen.** The ability of HCV core 178–induced CTLs to recognize endogenously synthesized CYP2A6 antigen was studied using the AHH-1 TK<sup>+/–</sup> cell line transfected with CYP2A6 (h2A3), and cells transfected with the unrelated CYP2D6 (h2D6) as target cells in 4- and 8-h cytotoxicity assays. Killing of CYP2A6-transfected cells by CTL lines from donor 2 was higher than lysis of the parental cell line AHH-1 TK<sup>+/–</sup> or control cells transfected with CYP2D6 (Fig. 4), suggesting that naturally processed CYP2A6 8–17 peptide is generated by the proteolytic machinery and presented on the HLA-A2 molecule.

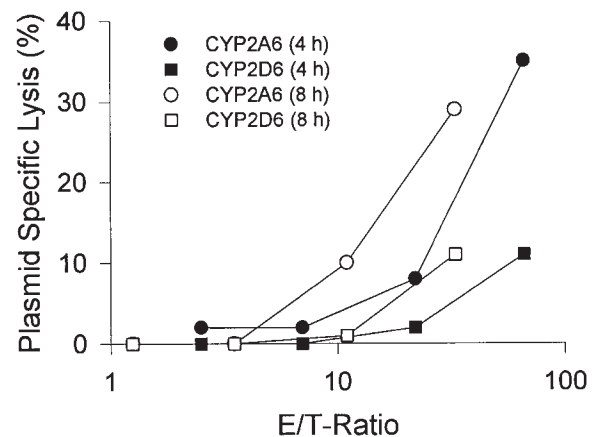
## Discussion

The HCV core 176 peptide has been described as a target epitope for CTLs that are present in the peripheral blood of patients with chronic HCV infection (10). This peptide is processed and presented via the endogenous MHC class I pathway (11). We used PBMCs from healthy, HCV-seronegative individuals as well as from patients with chronic HCV infection to induce primary CTL responses against this epitope and two homologous CYP-derived peptides. CTLs induced with the HCV core 178 peptide not only recognized the inducing HCV peptide, but also showed autoreactivity, lysing targets presenting CYP-derived self-peptides and target cells stably transfected with a plasmid coding for the whole CYP2A6 protein, showing that the CYP epitope is also presented via the endogenous MHC class I pathway. Among the CTLs we could distinguish three

different functional types in the same individual: cells recognizing HCV core 178 only, cells with cross-reaction between HCV core 178 and CYP2A7, and cells recognizing HCV core and both CYP epitopes, indicating a polyclonal response against HCV core 178 with a distinct hierarchy.

The induction of self-reactive CD8<sup>+</sup> CTLs by a viral epitope is compatible with molecular mimicry (resemblance of pathogen and host antigens), a mechanism that has been described mainly at the level of antibodies and CD4<sup>+</sup> T cells (16–18). Molecular mimicry at the level of T cells has been implicated in human autoimmune diseases such as multiple sclerosis (19, 20), rheumatoid arthritis (21), and myocarditis (22), as well as in herpes stromal keratitis (23). A recent study also describes cross-reactivity of CD8<sup>+</sup> T cells specific for a myelin-derived peptide with a *Saccharomyces cerevisiae* peptide (24). In this case, cross-reactive CTLs could only be induced with the self-peptide and not with the *Saccharomyces* antigen.

In our study we could not induce CTLs with the CYP-derived self-peptides, although the same self-peptides were rec-



**Figure 4.** Recognition of endogenously processed cytochrome P450. Cross-reactive CTL lines derived from donor 2 were tested for their ability to lyse AHH-1 TK<sup>+/–</sup> target cells transfected with CYP2A6 (circles) or CYP2D6 (squares). Shown is the plasmid-specific lysis: lysis of transfected cells – lysis of untransfected cells in a 4- (filled symbols) or 8-h (open symbols) cytotoxicity assay. The 4-h assay was performed in the presence of K562 cells.

ognized by CTLs induced with the HCV core 178 epitope. This indicates different T cell activation properties of the self-epitopes compared with the HCV peptide as described for altered peptide ligands (APLs), which are analogues of immunogenic peptides with amino acid substitutions inducing different effects in CTLs. APLs can act as agonists leading to full activation of T cells, partial agonists inducing only a reduced T cell response, or even antagonists inhibiting a response against the unaltered immunogenic epitope (33, 34). The molecular mechanisms of APL-induced partial T cell activation are still a matter of debate (35). Complexes of the native ligand or APLs with MHC molecules binding to the TCR can induce different intracellular signals in T cells (36–38), either by different oligomerization of necessary molecules (CD3, CD8, or other molecules), or by a failure of the APL to induce a required conformational change in the TCR. Other studies state that the level of T cell activation is dependent on the number of TCRs triggered in a process of serial engagement of many TCRs by a few peptide–MHC complexes, allowing a single CTL to generate different biological responses (39). Although specific cytotoxicity is already detectable at very low peptide concentrations, IFN- $\gamma$  production and proliferation require higher concentrations corresponding to higher numbers of TCRs being triggered (40). This is in accordance with the finding that the immunogenicity of antigenic peptides strongly correlates with the stability of MHC–peptide complexes formed (28), indicating that a high number and a high stability of MHC–peptide complexes is essential to trigger a sufficient number of TCRs to fully activate naive T cells. Despite this, the final effect of affinity differences or kinetic changes on the multimolecular interactions during antigen recognition cannot be predicted, as there is no strict correlation between functional activity of the various peptides and their MHC binding efficiency and the affinity of the MHC–peptide complexes for the TCR (31).

We have shown that the signal induced by the unstable complexes of HLA-A2 and CYP-derived self-peptides is not sufficient to activate naive CTLs. In the same way there is evidence that the CYP peptides neither induce negative selection during thymic development nor lead to anergy, as we detected cross-reactive CTL precursors in the peripheral blood. On the other hand, the HCV core 178 peptide that forms stable MHC–peptide complexes is able to induce full activation including maturation of CTLs. In the activated cross-reactive CTLs, cytolytic functions can then be induced by the HCV peptide, and also by the CYP-derived APLs, as cytotoxicity requires a lower threshold of activation than proliferation. Once activated, a subpopulation of cross-reactive CTLs that shows no differences in recognition of the three peptide ligands, because they recognize the HCV core 178 and the CYP peptides at similar peptide concentrations, can be found.

The clinical observation that HCV infection is preceding the development of LKM-1–positive AIH is in accordance with our hypothesis that antiviral immune response has to predate autoimmunity (41, 42). Moreover, the presence of autoreactive T cells in the blood and the liver of AIH type 2 patients has been demonstrated, indicating a role for both

CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the pathogenesis of this disease (4, 5). Cross-reactive CTLs may contribute to liver cell damage by lysis of infected and uninfected hepatocytes during ongoing viral infection and by lysis of uninfected autoantigen-expressing hepatocytes after viral clearance. However, the presence of virus-inducible autoreactive CTLs alone is not sufficient to lead to typical AIH, as none of the tested HCV patients had markers for AIH (LKM-1 autoantibodies). Probably the presence of autoreactive CD8<sup>+</sup> as well as autoreactive CD4<sup>+</sup> helper T cells is required for the induction of AIH. LKM-1 antigen (CYP2D6)–specific CD4<sup>+</sup> T cells detected in AIH patients would be needed for the activation of B cells secreting the AIH marker LKM-1 autoantibodies. Furthermore, autoreactive CD4<sup>+</sup> T cells could maintain the autoreactive CD8 T cell response after viral clearance, comparable with chronic viral infections where CD4<sup>+</sup> T cells are essential for maintaining the CTL response (43–46).

In summary, several arguments underscore the biological relevance of our findings. First, the viral epitope as well as the self-epitopes are naturally processed and presented by human host cells. Second, the self-epitopes and the viral epitope are coexpressed and colocalize to the liver in natural HCV infection, and expression of CYP2A6 is even enhanced in HCV-infected livers (47). Third, the absence of CYP-inducible CTLs in the peripheral blood suggests that central or peripheral tolerance mechanisms are operational, indicating that the self-epitopes are presented to T cells *in vivo*. Fourth, the data presented show a high level of cross-recognition of virally induced CTLs against the self-peptides, suggesting that they can mediate liver cell damage to uninfected cells. Fifth, another line of evidence links HCV infection to autoimmune hepatitis type 2 (6). This disease has been described to occur subsequent to HCV infection (42) and, interestingly, in association with HLA-A2 (48). Typically, it is associated with the presence of autoantibodies directed against cytochrome P450 2D6 (3, 6), as well as autoreactive CYP2D6-specific T cells, suggesting the parallel occurrence of autoreactivity against cytochrome P450 at the level of B and T cells (4, 5). It can be hypothesized that liver cell damage mediated by virus-specific CTLs leads to the release of intracellular proteins like CYP2D6, uptake of these autoantigens by professional APCs, and autoantibody formation in the presence of autoreactive CD4<sup>+</sup> T and B cells. After viral clearance, the autoimmune disease is upheld by ongoing hepatocyte lysis by cross-reactive, HCV-induced CTLs maintained by autoreactive helper T cells. The coincidence of HCV, autoreactive B cells, and autoreactive CD8<sup>+</sup> and CD4<sup>+</sup> T cells is thus required for the induction of HCV-associated AIH. This would explain the relatively low frequency of AIH among HCV patients despite the high number of individuals with cross-reactive CTLs.

Our findings demonstrate the potential of HCV to induce autoreactive CD8<sup>+</sup> CTLs by a molecular mimicry of CYP2A6/2A7 by the core protein and therefore show a possible mechanism by which HCV may trigger AIH. Further studies analyzing the cross-reactivity pattern of HCV core-specific CTLs derived from untreated patients with active HCV-associated autoimmune hepatitis are required in order to establish this mechanism.

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