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Patients with Chronic Hepatitis C Have Circulating Cytotoxic T Cells Which Recognize Hepatitis C Virus-Encoded Peptides Binding to HLA-A2.1 Molecules

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Antiviral cytotoxic T lymphocytes (CTL) may play a role in clearance of hepatitis C virus (HCV)-infected cells and thereby cause hepatocellular injury during acute and chronic HCV infection. The aim of this study was to identify HLA-A2.1-restricted HCV T-cell epitopes and to evaluate whether anti-HCV-specific CTL are present during chronic hepatitis C. Peripheral blood mononuclear cells from four HLA-A2-positive patients with chronic hepatitis C and from two individuals after recovery from HCV infection were tested against a panel of HCV-encoded peptides derived from different regions of the genome, including some peptides containing HLA-A2.1 binding motifs. HLA-A2-negative patients with chronic hepatitis C as well as healthy HLA-A2-positive (anti-HCV-negative) donors served as controls. Peripheral blood mononuclear cells stimulated repeatedly with several HCV-encoded peptides (three in core, one in NS4B, and one in NS5B) yielded cytolytic responses. All four HLA-A2-positive patients with active infection had CTL specific for at least one of the identified epitopes, whereas two patients who had recovered from HCV infection had almost no CTL responses. Monoclonal antibody blocking experiments performed for two epitopes demonstrated a class I- and HLA-A2-restricted CTL response. CTL epitopes could partially be predicted by HLA-A2 binding motifs and more reliably by quantitative HLA-A2.1 molecule binding assays. Most of the identified epitopes could also be produced via the endogenous pathway. Specific CTL against multiple, mostly highly conserved epitopes of HCV were detected during chronic HCV infection. This finding may be important for further investigations of the immunopathogenesis of HCV, the development of potential therapies against HCV on the basis of induction or enhancement of cellular immunity, and the design of vaccines.

Hepatitis C virus (HCV), a single-stranded plus-sense RNA virus within the *Flaviviridae* family, causes persistent infections in at least half of infected patients (9, 32). These chronically infected patients usually have chronic liver disease which can lead to cirrhosis and hepatocellular carcinoma (51). The only therapy of proven benefit for this disease is alpha interferon, which leads to sustained improvement of liver disease and elimination of the virus in only 15 to 20% of treated patients (15, 34, 52, 54). The search for new therapies and vaccines will require a better understanding of the pathogenesis of HCV infection and of the immune responses which are critical in the prevention and resolution of infection. Cellular immune responses probably play an important role in chronic HCV infection with respect to mediating both cellular injury and viral clearance (3, 7, 29–31, 40, 55, 57). Recent studies demonstrate that major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTL) of patients with chronic hepatitis C recognize epitopes from different regions of both structural and nonstructural HCV proteins (7, 29–31, 55, 57). In these studies, CTL responding to HCV-encoded antigens have been identified in peripheral blood and liver-infiltrating lymphocytes.

MHC-encoded molecules bind peptides of processed proteins and present them on the surface of antigen-presenting cells (APC) for recognition by T-cell receptors (4, 20, 61, 62). The presence of allele-specific amino acid motifs has been demonstrated by sequencing of peptides eluted from MHC molecules (18). Sequence analysis of in vivo-processed peptides eluted from purified HLA-A2.1 molecules has recently led to the definition of peptide motifs which bind to HLA-A2.1 as having preferred anchor residues within a nona- or decapeptide (16, 25, 27, 63). Recently, the role of secondary anchor residues in peptide binding to HLA-A2.1 molecules also has become evident (27, 50, 53). An extended motif taking into account secondary anchors increases the predictability of HLA-A2.1-binding epitopes.

We have performed CTL assays using a panel of HCV-encoded peptides within the structural and nonstructural regions of the HCV polyprotein. The HCV polyprotein, which consists of 3,010 to 3,033 residues, is processed to yield at least 10 cleavage products whose order has been established as NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH

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TABLE 1. Clinical data, HLA-A types, and virological features of patients studied for CTL responses to HCV epitopes

Group[a]	Patient or controls	HLA-A type	Disease duration (yr)	ALT (fold above upper limit)[b]	HCV-RNA by PCR genotype[c]	Received interferon or ribavirin in past
A	Ha	2	8	2	Negative	No
	Se	2 23	16	6	1b	Yes
	Su	2 3	3	4	1a	Yes
	Vi	2 28	12	4	3	Yes
	Sch	3	8	4	1a	Yes
	We	3 33	8	6	1b	Yes
B	Hi	2 11	7	Normal	Negative	Yes
	Ve	2 3	10	Normal	Negative	Yes
	Mu	1	6	Normal	Negative	Yes
	So	1	5	Normal	Negative	No
	St	1 32	22	Normal	Negative	Yes
C	2 healthy controls	2		Normal		
	2 healthy controls	3		Normal		

[a] Patients in group A had chronic hepatitis C (elevated ALT, anti-HCV positive). All patients in this group had chronic active hepatitis histologically. Individuals in group B are likely to recover after chronic hepatitis C (normal ALT, PCR negative). No recent liver biopsy (>2 years) was available for patients with inactive disease. Group C included two healthy HLA-A2 positive and two healthy HLA-A3-positive controls (anti-HCV negative).

[b] Serum ALT (normal range, 6 to 41 U/liter).

[c] Names according to the classification of Simmonds et al. (58).

(36). We were particularly interested in knowing whether peptides harboring HLA-A2.1 anchor residues at position 2 and the C terminus served as epitopes, and whether peptides yielding positive results had a strong ability to bind to HLA-A2.1. Therefore, we selected two panels of peptides according to the capacity to bind well or moderately to HLA-A2.1 molecules measured in an in vitro binding assay (50, 53) or to contain T-cell epitopes predicted by amphipathicity (14, 38, 55).

In this study, we found that patients with chronic hepatitis C harbor CTL directed against a variety of HCV proteins. These epitopes could be partially predicted by peptide motif analysis and by in vitro HLA-A2.1 binding assays, which proved to be an efficient strategy to define epitopes. The results obtained in this study also suggest that CTL responses can be enhanced in patients with chronic HCV infection, indicating the potential for their use as therapeutic vaccines.

MATERIALS AND METHODS

Patients. Patients with chronic hepatitis C were selected from among those monitored at the National Institutes of Health for possible entry into trials of antiviral therapy or after being treated. Table 1 summarizes patient characteristics and history of treatment. Patients who were studied had not received alpha interferon or other antiviral treatment for at least 6 months. Serum alanine aminotransferases (ALT) activities were measured by a multichannel autoanalyzer.

PBMC. Peripheral blood mononuclear cells (PBMC) were obtained by lymphopheresis. HLA typing was performed by conventional methods by the clinical laboratories at the National Institutes of Health. Six HLA-A2-positive patients with chronic hepatitis C were selected; four had active disease, whereas two appeared to have resolved chronic infection, having had normal ALT levels and no HCV RNA in serum for more than 3 years after alpha interferon therapy. In addition, we obtained PBMC from HLA-A2-negative patients with chronic hepatitis C as well as from two HLA-A2-positive and two HLA-A2-negative healthy donors who tested anti-HCV negative.

Peptides. HCV peptides were prepared by solid-phase peptide synthesis, using standard fluorenylmethyloxycarbonyl chemistry on a Miligen Excel peptide synthesizer (Waters Associates, Milford, Mass.) or on an Applied Biosystems 430A peptide synthesizer as described in detail elsewhere (50, 53). One peptide panel was derived from NS4B-NS5B of the HCV genome (Table 2), which was selected by amphipathicity (14, 38, 55) and of which nine peptides (peptides 4, 5, 11, 15, 17, 19, 23, 25, and 26) contained HLA-A2.1 binding motifs. The sequences of NS4B-NS5B peptides were based on that of the HCV-1 (10), which corresponds to HCV isolate 1a according to the classification proposed by P. Simmonds et al. (58). The other peptides (see Table 5) were chosen because they were highly conserved, contained HLA-A2.1 binding motifs, and demonstrated intermediate (50 to 500 nM) or high (<50 nM) binding to HLA-A2.1. Ability to bind to HLA-A2.1 molecules was quantitated with an in vitro assay based on the inhibition of binding of a radiolabeled standard peptide to purified detergent-solubilized HLA-A2.1 molecules (50, 53).

Recombinant vaccinia viruses. A recombinant vaccinia virus expressing HCV core, E1, E2, and part of the NS2 region (vHCV 1-966) (20a) or a recombinant expressing part of NS2 and the entire NS3-NS5B region (vHCV 827-3011) (21) under control of the T7 promoter was used to infect concanavalin A-stimulated

TABLE 2. Peptides derived from the NS4B-NS5B region[a]

Peptide	Residues	Sequence
3	1958–1977	RRLHQWISSECTTPCSGSWL
4	1969–1988	TTPCSGSWLRDIWDWICEVL
5	1981–2000	WDWICEVLSDFKTWLKAKLM
6	2042–2061	GMRIVGPRTCRNMWSGTFP
7	2084–2103	RVSAEEYVEIRQVGDFHYVT
8	2089–2108	EYVEIRQVGDFHYVTGMTTD
9	2113–2132	PCQVPSPEFFTELDGVRLHR
10	2117–2136	PSPEFFTELDGVRLHRFAPP
11	2168–2183	VAVLTSMLTDPSHITA
12	2180–2195	ITAEAAGRRLARGSP
13	2265–2284	ERAISVPAEILRKSRRFAQA
14	2267–2286	AISVPAEILRKSRRFAQALP
15	2335–2354	LTESTLSTALAELATRSFGS
16	2348–2362	ATRSFGSSSTSGITG
17	2422–2437	MSYSWTGALVTPCAAE
18	2438–2455	EQKLPINALSNSLLRHHN
19	2477–2497	LQVLDSHYQDVLKEVKAAASK
20	2531–2550	HARKAVTHINSVWKDLLEDN
21	2535–2554	AVTHINSVWKDLLEDNVTPI
22	2583–2598	PDLGVRVCEKMALYDV
23	2593–2607	MALYDVVTKLPLAVM
24	2668–2683	QARVAIKSLTERLYVG
25	2701–2719	ASGVLTTSCGNTLTCYIKA
26	2721–2736	AACRAAGLQDCTMLVC
27	2749–2768	VQEDAASLRAFTEAMTRYSA
28	2757–2776	RAFTEAMTRYSAPPGDPPQP
29	2822–2834	HTPVNSWLGNIIM
31	2866–2880	EIYGACYSIEPLDLP

[a] Peptides were used in the experiments shown in Table 3. The sequences of these peptides were based on that of the HCV-1 isolate (10). Peptides with HLA-A2.1 binding motifs and the corresponding potential anchor residues are underlined. Leucine (L) and methionine (M) at position 2 and leucine (L), valine (V), or isoleucine (I) at position 9 or 10 were identified as preferred anchor residues (27).

(ConA) blast target cells along with a vaccinia virus expressing the T7 RNA polymerase (vT7) (21) for presentation of endogenously processed HCV proteins. The amino acid sequences of the vHCV recombinants are derived from the HCV-H cDNA clone, which corresponds to the HCV 1a isolate.

CTL generation. After Ficoll-Hypaque (Pharmacia) separation, we stimulated PBMC (3×10^6 per 48-well culture plate) in the presence of 10 μM peptide at days 1, 7 or 8, and 14 in complete T-cell medium (CTM; 1:1 mixture of RPMI 1640 and EHAA medium containing 10% fetal bovine serum, 4 mM L-glutamine, 5 μg of gentamicin per ml, and 50 μM 2-mercaptoethanol). CTM containing human interleukin-2 (IL-2; 10% [vol/vol]; Boehringer Mannheim) was added every third day except when cultures were stimulated with peptide. PBMC were stimulated with peptide weekly for a maximum of six times. For the first two stimulations, we used irradiated (3,000 rad) autologous PBMC and thereafter allogeneic irradiated PBMC (10^6 per well) as feeder cells and autologous irradiated ConA blasts (2×10^5 per well) as APC. ConA blasts were obtained by stimulating PBMC with concanavalin A (2 μg/ml; Sigma) for 4 to 5 days and then changing the medium with CTM containing recombinant IL-2 (50 U/ml) and recombinant IL-6 (1 U/ml). ConA blasts were restimulated with concanavalin A every 8 to 10 days.

CTL assay. Cytolytic activity of CTL was measured in a 6-h assay with ^{51}Cr-labeled targets. As target cells, we used ConA blasts labeled with ^{51}Cr in the presence or absence of specific peptides or infected with a recombinant vaccinia virus (see below). For testing the peptide specificity of CTL, effector cells and ^{51}Cr-labeled target cells were mixed in the presence or absence of peptide (10 μM). To test endogenous processing of epitopes, target cells were infected (multiplicity of infection [MOI] of 5) with either vHCV 1-966 or vHCV 827-3011) along with vaccinia virus expressing the T7 polymerase (MOI of 5). As controls, targets cells were infected with the latter vaccinia virus alone (MOI of 10). Infected cells were incubated for 1 h at 37°C in 5% CO_2 and washed twice thereafter. Cells were then labeled with ^{51}Cr (50 μCi/5×10^5 cells per well in a 24-well plate) and incubated overnight. The next day, the cells were washed three times before being used in a 6-h ^{51}Cr release assay. The lytic activity of CTL was tested in CTM containing 2% fetal bovine serum in triplicate with effector target cell (E/T) ratios of 70:1, 23:1, and 8:1, unless otherwise indicated, using 5×10^3 to 1×10^4 target cells per 96-well plate. The percent specific lysis was calculated as $100 \times$ [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum ^{51}Cr release was determined from supernatants of cells that were lysed by addition of 2% Nonidet P-40. Spontaneous release was measured in supernatants from target cells incubated without effector cells, and assays were excluded from analysis if the spontaneous release value was >30% of maximum. Significant cytotoxicity was defined as >15% specific lysis.

Blocking of CTL response by antibodies. CTL responses were tested in the presence of an anti-class I (W6/32), anti-class II (L227) (both obtained from the American Type Culture Collection), or anti-HLA-A2 (BB7.2) (47) monoclonal antibody added to the 96-well plates used for the CTL assay. The final dilutions of the hybridoma culture supernatant in the assay were 1:4 and 1:8.

RESULTS

PBMC were obtained by lymphopheresis from 15 individuals categorized into three groups on the basis of hepatitis C status (Table 1). Group A consisted of six patients with chronic hepatitis C (anti-HCV positive, raised ALT) and included four patients with HLA-A2. Group B consisted of five patients with resolved or inactive hepatitis C (anti-HCV positive but normal ALT and no HCV RNA in serum) and included two patients with HLA-A2. Group C consisted of four healthy controls (normal ALT, anti-HCV negative), two of whom had HLA-A2.

To test whether peptide could stimulate PBMC to lyse autologous target cells which had been sensitized with corresponding peptides, we first used a panel of 28 peptides derived from the NS4B-NS5B region of HCV (Table 2) and selected on the basis of amphipathicity (14, 38, 55). Several peptides contained potential HLA-A2.1 binding motifs with leucine and valine as the preferred anchor residues (i.e., xLxxxxxx[x]V or xLxxxxxx[x]L). Other peptides contained the less optimally tolerated anchor residues for binding to HLA-A2.1 (27). After two stimulations with NS4B-NS5B-derived peptides, PBMC from three of four HLA-A2-positive patients with active disease lysed autologous target cells labeled with peptide NS5B peptide 26, which contained an HLA-A2.1 binding motif (Table 3). Specific lysis of 15% or more was considered significant. Although several NS5-derived peptides contained an HLA-A2.1 binding motif, no other peptides induced CTL activity (data not shown). PBMC of HLA-A2-negative individuals tested with the entire NS4B-NS5B panel or healthy donors tested with HLA-A2 motif peptides all yielded <5% specific lysis in the CTL assays.

NS5B peptide 26 was a 16-mer and contained several amino acid sequence motifs which could possibly account for the stimulatory epitope. Accordingly, nine shorter peptides, 8 to 11 amino acids in length, were synthesized such that each peptide had either a preferred (L) or a tolerated (A) amino acid res-

TABLE 3. CTL activity against NS4B-NS5B peptides[a]

Group	Patient or controls	HLA type at indicated locus			^{51}Cr-release (% lysis)[b]	
		A	B	C	Peptide labeled	No peptide
A	Ha	2	7 62	3 7	33.7	8.3
	Se	2 23	45 49	6 7	20.6	5.6
	Su	2 3	7 47	2 7	13.1	4.4
	Vi	2 28	7 35	4 15	22.1	3.9
	Sch	3	7	7	8.2	3.7
	We	3 33	35 70	2 6	6.1	4.0
B	Hi	2 11	35 51	4	7.9	5.8
	Ve	2 3	7 50	6 7	8.6	2.2
	Mu	1	8 62	3	7.1	6.5
	So	1	8	7	9.3	8.1
	St	1 32	44 57	6	6.4	7.6
C	2 healthy controls	2	ND[c]	ND	4.0, 3.8	4.6, 5.0
	2 healthy controls	3	ND	ND	6.2, 9.7	5.2, 7.3

[a] PBMC of HLA-A2-positive patients with active (group A) and inactive (group B) disease and of anti-HCV-negative healthy controls (group C) were stimulated twice in vitro with peptides (10 μM) derived from NS4B-NS5B (as described in Materials and Methods). Other HLA class I loci (HLA-B and HLA-C) are also shown.

[b] Stimulated PBMC were tested against autologous ConA blast target cells in the presence (10 μM) or absence of the peptide at E/T ratios of 50:1 (shown), 23:1, and 8:1 in triplicate (standard error of the mean, <5%). Spontaneous release was below 25% for all target cells, and we did not observe peptide toxicity against targets. Results for NS5B peptide 26 are shown (one of two similar experiments). The remaining NS4B-NS5B peptides were tested in all patients except Hi and Se, but CTL responses were absent (<5%).

[c] ND, not determined.

TABLE 4. Potential CTL epitopes within NS5B peptide 26[a]

NS5B peptide 26-derived peptides	No. of amino acids	Sequence[b]	HLA-A2.1 binding (50% inhibitory concn [nM])[c]
26.01	8	**A**ACRAAG**L**	>50,000
26.02	8	**A**AGLQDC**T**	>50,000
26.03	9	**A**AGLQDC**TM**	>50,000
26.04	10	**A**AGLQDC**TML**	>50,000
26.05	11	**A**AG**L**QDC**TL**V	50,000
26.06	9	G**L**QDCTML**V**	23
26.07	9	R**A**AGLQDC**T**	>50,000
26.08	10	R**A**AGLQDC**TM**	>50,000
26.09	11	R**A**AGLQDC**TL**	>50,000

[a] NS5B peptide 26 was analyzed for harboring HLA-A2.1 binding motifs, and thereafter nine shorter peptides from the region between residues 2721 and 2735 were synthesized according to potential HLA-A2.1 binding motifs with either preferred or tolerated anchor residues at position 2 and the C terminus.

[b] Boldface underlined amino acids define preferred (optimal) anchor residues at positions 2 and 9 or 10. Boldface letters not underlined depict tolerated amino acids at these positions.

[c] Tested in vitro in an HLA molecule binding assay (50, 53).

FIG. 1. CTL from three patients were stimulated four times with the original NS5B peptide 26 (16-mer). Target cells were sensitized with NS5B peptide 26-derived peptide 26.04, 26.05, or 26.06 (Table 4), and lysis was tested in a ^{51}Cr release assay. E/T ratios were set at 50:1, 17:1, and 6:1 in triplicate. Lysis is shown with an E/T ratio of 50:1 (standard error of the mean, <5% lysis). AA, amino acids.

idue at position 2 and a preferred (L or V) or a tolerated (T or M) amino acid residue at the C terminus (Table 4). These same peptides were tested for the ability to block binding of a known HLA-restricted peptide to purified HLA-A2.1 molecules in vitro (50, 53). Despite containing the established HLA-A2.1 binding motifs, only one of the nine peptides demonstrated efficient binding to HLA-A2.1 in vitro; i.e., 23 nM peptide 26.06 inhibited at least 50% binding of a radiolabeled standard peptide to HLA-A2.1 molecules. Peptide 26.05 bound weakly, whereas all seven of the other peptides did not have measurable binding to HLA-A2.1 molecules. Subsequently, we tested whether CTL lines repeatedly stimulated with the original peptide 26 lysed autologous target cells sensitized with peptides 26.04, 26.05, and 26.06. Only target cells labeled with the strongly binding peptide (26.06) were lysed (Fig. 1), demonstrating a good correlation of in vitro testing for binding to HLA molecules with results obtained in the ^{51}Cr release assay.

Because the results of in vitro binding to HLA-A2.1 molecules predicted the ability to stimulate CTL activity, we further tested a panel of nine peptides derived from various regions of the HCV polyprotein (Table 5). This panel of peptides was selected after scanning the HCV polyprotein for amino acid sequences with motifs for HLA-A2.1. Corresponding peptides were synthesized, and nine peptides which demonstrated either high (<50 nM) or intermediate (50 to 500 nM) binding to HLA-A2.1 molecules in vitro were chosen for further testing. With these nine peptides, we tested four HLA-A2-positive patients with chronic active hepatitis C and two individuals who seemed to have recovered from disease (Tables 1 and 5). After four or six stimulations, significant CTL responses were found with five peptides (1073.07, 1013.10, 939.20, 1073.05, and 1073.06); i.e., PBMC stimulated repeatedly with one of the five peptides lysed autologous target cells sensitized with the corresponding peptide. Not all patients had PBMC recognizing all epitopes. However, in all HLA-A2-positive patients with active disease, CTL specific for at least one HCV epitope could be detected.

TABLE 5. CTL responses against a panel of peptides with high or intermediate binding to HLA-A2.1[a]

Peptide[b]					HLA-A2.1 binding (50% inhibitory concn [nM])	^{51}Cr release (% specific lysis)[d]					
Derivation	Name	Residues	Sequence	Conservation data[c]		Ha	Se	Su	Vi	Hi	Ve
Core	1073.07	35–44	YLLPRRGPRL	16/16	125.0	<5	ND	<5	29.9 (39/9)	ND	8.5
	1013.10	132–140	DLMGYIPLV	16/16	80.0	<5	ND	<5	16.4 (21/5)	ND	<5
	939.20	178–187	LLALLSCLTV	16/16	357.1	44.7 (56/14)	ND	<5	41.2 (50/9)	ND	<5
E2	1013.12	686–694	ALSTGLIHL	16/16	312.5	9.7	ND	<5	13.4	ND	<5
	1090.18	725–734	FLLLADARV	16/16	17.9	<5	ND	<5	<5	ND	ND
NS3	1013.02	1585–1593	YLVAYQATV	14/16 (J6 + J8)	59.0	<5	ND	8.6	<5	ND	<5
NS4B	1073.05	1807–1816	LLFNILGGWV	14/16 (J6, J8)	4.2	32.6 (38/5)	28.3 (37/9)	42.2 (51/9)	68.1 (80/12)	<5	6.3
	1073.06	1851–1859	ILAGYGAGV	14/16 (J6, J8)	115.5	46.6 (53/6)	ND	27.0 (37/10)	39.6 (48/9)	ND	21.2 (26/5)
NS5B	1090.22	2578–2587	RLIVFPDLGV	13/16 (JK1, J6, J8)	56.7	10.2	ND	<5	5.9	ND	5.3
	26.06	2727–2735	GLQDCTMLV	4/16	23.0	21.8 (29/7)	31.2 (35/4)	10.3	26.6 (31/4)	8.4	<5

[a] CTL responses after six stimulations are shown in four patients with active disease (Ha, Se, Su, and Vi) and two individuals who recovered and have no virological or biochemical signs of ongoing infection (Hi and Ve). Stimulation of PBMC, labeling of target cells, and ^{51}Cr release assays were performed as described in Materials and Methods. PBMC were tested against target cells in the presence or absence of specific peptide.

[b] Peptides were chosen because they were highly conserved, contained HLA-A2.1 binding motifs, and demonstrated intermediate or high binding to HLA-A2.1. NS5B peptide 26.06 was found after cytotoxic activity was obtained with NS5B peptide 26 when a panel of 28 NS4B-NS5B peptides was tested.

[c] Alignment of 16 full-length HCV polyprotein sequences was analyzed with the Genetics Computer Group suite of programs (version 7). These 16 sequences included HCV-H-CMR (12), HCV-1 (10), HCV-H-GI (26), HCV-J1 (GenBank accession number D10749), HCV-JT (60), HCV-JTB (60), HCV-J483 (44), HCV-J491 (44), HCV-JA (28), HCV-BK (59), HCV-TW (8), HCV-JK1 (GenBank accession number S18030), HCV-Unkcds (GenBank accession number M96362), HCV-N (22), HCV-J6 (46), and HCV-J8 (45). The conservation data show how many strains are conserved compared with the tested peptide. NS5B peptide 26.06 shows conservation between strains HCV-H, HCV-1, HCV-J1, and HCV-H-CMR and is 100% conserved regarding the anchor residues.

[d] Mean of triplicate samples (standard error of the mean, <5%) and representative of at least two independent experiments. Specific lysis above 15% was considered significant. In parentheses is shown percent killing on peptide-coated versus control targets for which specific lysis was equal to or more than 15%. Lysis is demonstrated at an E/T ratio of 70:1. Lysis of uncoated target cells was below <15% when not indicated.

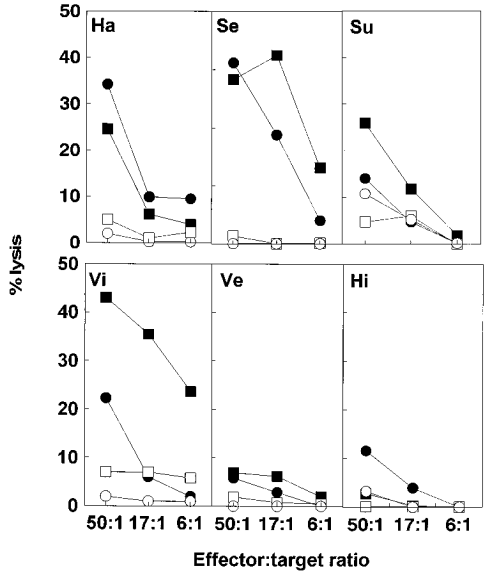

FIG. 2. PBMC were stimulated four times with peptide NS4B peptide 1073.05 or NS5B peptide 26, and cytotoxicity was measured thereafter against target cells labeled with the corresponding peptide (5 μM) or in the absence of peptide with indicated E/T ratios. PBMC were stimulated with NS4B peptide 1073.05 and tested against target cells in the presence of NS4B peptide 1073.05 (■) or absence of peptide (□). PBMC were stimulated with NS5B peptide 26 and tested in the presence (●) or absence (○) of the corresponding peptide. Patients Ha, Se, Su, and Vi have chronic active hepatitis C; individuals Ve and Hi have recovered from HCV infection.

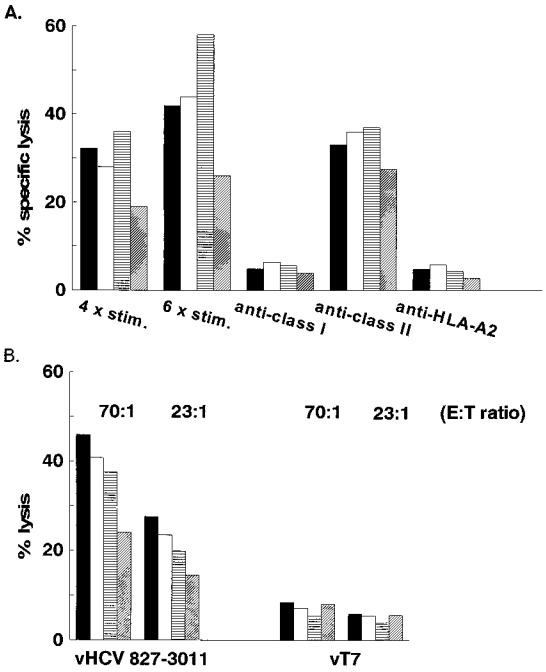

FIG. 3. (A) PBMC of patients Ha and Vi were stimulated four or six times with the indicated peptides (■, patient Ha, NS4B peptide 1073.05; ▤, patient Vi, NS4B peptide 1073.05; □, patient Ha, NS5B peptide 26; ▨, patient Vi, NS5B peptide 26), and the cytotoxicity of these T-cell lines was measured against autologous ConA blasts sensitized with the same peptide or in the absence of the peptide. Lysis was also tested in the presence of a hybridoma culture supernatant containing an anti-class I (W6/32), anti-class II (L227), or anti-HLA-A2 (BB7.2) monoclonal antibody in the CTL assay (final dilution, 1:4). Specific lysis, i.e., lysis of ConA blasts sensitized with the peptide, is shown. (B) The effector cells were the same as those used for panel A. As target cells, ConA blasts were infected with vHCV 827-3011, coding for part of NS2 and the entire NS3-NS5B region. As control targets, ConA blasts were infected with vaccinia virus expressing the T7 polymerase alone.

The target cell lysis obtained after two stimulations of PBMC with peptides was usually below 20% specific lysis, even when peptides with high binding capacity were used. Results with two peptides, one in NS4B and one in NS5B, demonstrated that lytic capacity was further enhanced after four and six stimulations (Fig. 2 and 3A). No lysis was detected even after six stimulations in cases in which there was no lysis after two stimulations. Two individuals with resolved infections (Hi and Ve) did not lyse autologous target cells labeled with the most efficient peptides (NS4B peptide 1073.05 and NS5B peptide 26) despite four and six stimulations (Fig. 2 and Table 5). No lysis was found with these peptides in healthy anti-HCV-negative, HLA-A2- or HLA-A3-positive donors after four stimulations (data not shown). Hence, the repeated stimulation seemed to enhance the lytic activity probably by increasing the frequency of specific CTL in the culture, allowing a clearer discrimination of CTL responses. Taken together, these results demonstrated that CTL specific for a broad variety of epitopes within the core, NS4B, and NS5B regions exist in detectable levels in patients with chronic hepatitis C but not in individuals who recovered from disease. It is noteworthy that despite repeated stimulation, no CTL could be detected against E2 peptides 1013.12 and 1019.18, the latter demonstrating high binding to HLA-A2.1 molecules, peptide 1013.02 within NS3, or peptide 1090.22 within NS5B.

We further tested whether the defined epitopes were intracellularly processed and presented for CTL by APC. Target cells were infected with recombinant vaccinia virus vHCV 827-3011, expressing most of nonstructural proteins of HCV, including NS4B-NS5B. PBMC from HLA-A2-positive patients with chronic hepatitis C stimulated with NS4B peptide 1073.05 and NS5B peptide 26 yielded specific lysis of target cells infected with vaccinia virus expressing most of the nonstructural regions of the viral polyprotein, indicating that these antigens could be endogenously processed and expressed with MHC class I molecules (Fig. 3B). Target cells infected with vaccinia virus not expressing HCV proteins were not lysed by these CTL. Endogenous processing was also demonstrated for peptides derived from the core region (1073.07, 1013.10, and 939.20), using a recombinant vaccinia virus expressing core, E1, E2, and part of the NS2 region (vHCV 1-966) (Fig. 4). No lysis was obtained by using vHCV 827-3011 with PBMC which were stimulated with NS4 peptide 1073.06 despite lysis of peptide-labeled target cells and intermediate binding to HLA-A2.1 molecules (Fig. 4). To determine the amount of peptide needed to sensitize target cells, ConA blasts were incubated with different concentrations of two peptides to which most of the patients with active disease reacted (NS4B peptide 1073.05 and NS5B peptide 26). For both tested peptides, 5 μM was needed to sensitize ConA blast serving as target cells (data not shown).

Blocking of the CTL responses with anti-class I, anti-class II, and anti-HLA-A2.1 antibodies demonstrated that the response was class I and HLA-A2.1 restricted (Fig. 3A), as lysis was nearly abrogated with anti-class I and anti-HLA-A2 antibodies but almost identical when anti-HLA class II antibodies were coincubated during the ^{51}Cr release assay.

DISCUSSION

In this study, we found that a variety of MHC class I-restricted CTL were detectable in peripheral blood of HLA-A2-

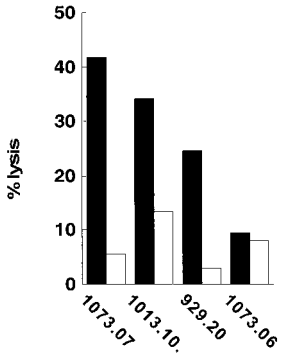

FIG. 4. PBMC of patient Vi were stimulated four times with peptide 1073.07 (core), 1013.10 (core), 939.20 (core), or 1073.06 (NS4B), and the cytotoxicity of these T-cell lines was measured against autologous ConA blasts infected with the corresponding vHCV recombinant (filled columns), i.e., either vHCV 1-966, coding for core, E1, E2, and part of NS2, or vHCV 827-3011, coding for part of NS2 and the entire NS3-NS5B region. As controls (open columns), we used the recombinant vaccinia viruses expressing unrelated parts of HCV (e.g., targets infected with vHCV 827-3011 served as controls for T-cell lines stimulated with core peptide 939.20).

positive patients with chronic hepatitis C. These CTL responses were directed against epitopes containing HLA-A2.1 binding motifs with preferred anchor residues at position 2 and the C terminus. High or intermediate binding to HLA-A2.1 molecules in vitro was predictive of active CTL epitopes. However, not all peptides with HLA-A2.1 binding motifs or HLA-A2.1 binding were found to function as epitopes. Patients with active HCV infection harbored CTL that recognized a broad variety of epitopes, whereas individuals who recovered seem to have declining or absent CTL activity.

Importantly, in these studies we had to stimulate PBMC with peptide repeatedly to obtain CTL with good capacity to lyse autologous target cells sensitized with peptide or infected with a recombinant vaccinia virus expressing HCV proteins that included the corresponding amino acid sequence. CTL responses were absent in two healthy HLA-A2-positive donors without evidence of HCV infection, indicating that the response seen in patients was due to T cells that had already been primed. In vitro induction of antigen-specific CTL is possible in normal individuals; however, use of mutant APC such as the RMA-S and T2 cell lines, which are antigen processing defective (13, 24), professional APC such as dendritic cells (2, 39), or a specialized protocol in which autologous APC are loaded with large amounts of peptide is required (6, 66). In fact, with a specialized protocol, some of the peptides used in the present study were capable of in vitro induction of primary, HCV-specific CTL from the PBMC of normal individuals (65). It is unlikely that the CTL responses seen in patients with active disease were due to activation of naive T cells (11), as CTL responses were measured after two stimulations. Also, two individuals who apparently recovered from infection had weak or no CTL responses, consistent with absent or very low precursor frequencies.

Selection of peptides based on amphipathicity resulted in the identification of 1 of the 28 peptides tested as capable of generating a CTL response. When a second set of peptides was selected on the basis of HLA-A2.1 binding, five of the nine peptides (56%) tested were recognized by CTL; four of these peptides were also endogenously processed. Thus, an approach to identify CTL epitopes based on in vitro binding to HLA molecules appears to be more effective. The results of screening the HLA-2.1-binding peptides confirm our recent study describing the core peptide 1013.10 as an epitope (57). Several peptides within NS5 contained HLA-A2.1 motifs, but only one peptide served as an epitope, further underlining the fact that the presence of HLA-A2.1 binding motifs could only partially predict the presence of CTL epitopes. Secondary anchor positions in addition to primary anchors seem to be important for binding to HLA molecules, as the presence of primary anchor motifs may be necessary but not sufficient for binding (27, 37, 50, 53). Epitopes identified in this study (three within core, one within NS4B, and one within NS5B) contained preferred, i.e., optimal, amino acid residues at both anchor positions, confirming that peptide binding with high affinity to a particular HLA-A molecule is limited (27).

The results obtained with the panel of HLA-A2.1 high- and intermediate-binding peptides revealed that not all binding peptides could serve as CTL epitopes for these patients (e.g., E2 peptide 1090.18 and NS4 peptide 1013.02). E2 peptide 1090.18 also was not found to induce CTL responses in HLA-A2-transgenic mice or in Japanese patients with chronic hepatitis C (56). The reason for the absence of CTL responses despite avid in vitro binding is not clear. It is possible that peptide sequences are not produced by intracellular processing during HCV infection. Another explanation is that the CTL response to these sequences is absent because of tolerance due to their homology to self proteins (7, 43). However, before conclusions can be reached with regard to the possible causes of lack of recognition, a larger patient sample must be analyzed.

It should be stressed that two of the patients with active disease studied here did not recognize all identified epitopes. The variation in responsiveness to different epitopes that bind to HLA-A2.1 molecules also deserves analysis. Interindividual differences in processing of viral proteins and tolerance induction (as mentioned above) may partially explain these differences. For example, polymorphism in self proteins may result in different tolerance patterns. It is possible that patients with active disease and different genotypes did not recognize epitopes because different viral proteins were processed. However, most of the peptides in Table 5 were highly conserved among HCV isolates and were also likely to have been expressed in patients who had apparently cleared the virus. Only the epitope in NS5B derived from NS5B peptide 26.06 demonstrated some sequence variability among isolates but was still conserved between HCV-1a and 1b isolates, the major genotypes found among U.S. patients. In addition, no amino acid changes in the anchor residues at position 2 and the C terminus were found within this epitope among 16 different isolates. Therefore, strain variation or a CTL escape mutant virus, such as shown with other viruses (48, 49), is unlikely a cause for lack of CTL responses to specific epitopes. Another possibility for the variation in CTL responses is that corresponding CTL were induced during early stages of HCV infection but because of exhaustive differentiation were clonally deleted later, allowing virus to persist (1, 5, 41, 67). Other possibilities may include the absence of T-cell receptors for these epitopes in some patients, differences in other HLA loci influencing the strength of CTL responses, and finally sequestration of some CTL and other immune cells in the liver (30, 40).

Two individuals who recovered did not demonstrate lysis when their PBMC were assayed against target cells labeled with NS4B peptide 1073.05 or NS5B peptide 26. One may argue that CTL were not circulating in peripheral blood but were present in the liver. This is unlikely since both patients had inactive liver disease and their ALT levels were persistently normal for more than 2 years. It is possible that not

enough antigen was present after elimination of HCV to stimulate and maintain CTL responses. It remains to be elucidated if long-term CTL memory in HCV infection requires the presence of HCV antigen. Recent findings suggest that antivirally protective CTL memory is governed by persisting antigen (42), whereas other studies indicate that CTL long-term memory persists in the absence of priming antigen (23, 35). Precursor CTL might be found in the liver or lymphoid tissue, but the absence of any CTL responses identifiable in PBMC suggests that the frequency is low or, as mentioned above, CTL are present but restricted to HLA-B or HLA-C. The occurrence of reinfection with HCV in humans and chimpanzees despite the presence of a humoral immune response (19, 33) could be due in part to the decline of the specific cellular immune responses.

With this strategy to identify epitopes, i.e., screening the HCV polyprotein for conserved HLA-A2.1 epitope motifs for CTL, assays of peptide binding to HLA-A2.1, and ^{51}Cr release assays, we confirmed the description of an epitope in core in mice and humans (1013.10) (57). HLA-A2-restricted CTL recognizing epitopes within HCV core and NS4 regions but not in the E1 or E2 region were described recently (7). However, HLA-B7-restricted liver-infiltrating liver lymphocytes recognizing an HCV E2 peptide were described (30). This finding demonstrates that CTL which are HLA-B or -C restricted or the alternative HLA-A allele may play an important role in the antiviral immune response (29). Interestingly, the NS4 region was also found to be very immunogenic for peripheral as well as liver-derived $CD4^+$ T cells (3, 40), indicating a possible role of class II-restricted effector cells. Taken together, our and earlier results demonstrate that a broad variety of HCV epitopes are recognized by CTL (7, 17, 29–31, 55). With respect to comparison of the anti-HCV CTL response with the anti-HBV CTL response, it is worth mentioning that CTL responses were not always found in the peripheral blood of patients with chronic HBV infection. The reason for this discrepancy is not clear, but it may be that in patients with chronic HBV infection, T-cell responses are not as vigorous or may be more restricted to the liver than during chronic (ongoing) HCV infection (7).

Although patients with chronic hepatitis C seemed to have a broad CTL repertoire (7, 29–31, 55, 57) to HCV, they were still unable to resolve the chronic infection. It is possible that CTL were not present in high enough numbers to eliminate HCV but are capable of causing damage via lysis of hepatocytes expressing HCV proteins. Broadening the CTL immune response could help eliminate the virus, but it also might enhance immunopathology, with the infected hepatocytes serving as targets for the activated T cells. Nevertheless, the presence of a broad spectrum of CTL responses to HCV and the possibility of expanding these CTL populations in vitro provide a basis for immunotherapeutic interventions to enhance T-cell immunity in this disease. It remains to be shown whether CTL responses can be enhanced or, in particular, induced in vivo in patients with active disease in whom antigenic load should be high enough for CTL responses and in whom some CTL lines may have been clonally deleted. However, the use of peptides to induce CTL responses in humans has been recently demonstrated in the hepatitis B virus system (64). Thus, a possible therapeutic approach to increase HCV-specific CTL activity to entirely eliminate virus could be contemplated. It is clear that a better understanding of the mechanisms and role of T-cell immunity in acute and chronic hepatitis C will be crucial to progress in both the prevention and treatment of this important disease.

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