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Characterization of an immunologically conserved epitope from hepatitis C virus E2 glycoprotein recognized by HLA-A2 restricted cytotoxic T lymphocytes

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***Background/Aims*: Identification of epitopes recognized by cytotoxic T lymphocytes (CTL) in hepatitis C virus (HCV) proteins is of importance because they can be used for vaccination, treatment of infection or monitoring of immune responses. Our purpose was to characterize new CTL epitopes in HCV structural proteins.**

***Methods*: Peptides were synthesized and tested in HLA-A2 binding assays. Binder peptides were used to stimulate peripheral blood mononuclear cells from HCV$^+$ patients and controls, and activity measured in chromium release and ELISPOT assays.**

***Results*: Twenty binder peptides were found, and stimulation of HCV$^+$ patient cells with nine peptides showing high binding ability led to the growth of CD8$^+$ CTL recognizing peptide E2(614-622) in association with HLA-A2. Peptide E2(614-622) was recognized by 30% of HLA-A2$^+$ patients with chronic HCV infection, but no responses were observed in control groups. Five peptides derived from region E2(614-622) from 26 different viral isolates bound to HLA-A2 molecules, and all of them but one, containing Phe at position 622, were recognized by E2(614-622) specific CTL.**

***Conclusions*: These results show that peptide E2(614-622) belongs to a highly conserved region of HCV E2, and might be a good candidate to induce anti-HCV CTL responses in HLA-A2$^+$ subjects.**

***Keywords*: Hepatitis C virus; Cytotoxic T lymphocytes; Epitope; HLA binding**

1. Introduction

Hepatitis C virus (HCV) is a single stranded RNA virus responsible for the majority of non-A non-B hepatitis [1]. Infection with HCV frequently evolves to chronicity, and ultimately to cirrhosis and hepatocellular carcinoma [2]. After infection, patients elicit a wide range of antibodies against viral proteins [3], which do not seem to be relevant to clear infection. CD4$^+$ T-cell responses are also elicited [4–10], although they are very weak or undetectable in chronically infected patients. As in other viral infections, CD8$^+$ cytotoxic T lymphocytes (CTL) have been detected in HCV patients [11–17], and recent reports show their importance in viral clearance in the acute phase of the disease [18] and in HCV-exposed seronegative donors [19,20]. CTL usually eliminate infected cells after recognition of viral antigens presented as short peptides in the HLA molecules. These peptides are produced after processing of viral proteins and they are presented by HLA molecules on the cell surface [21]. The use of synthetic peptides has allowed the characterization of these epitopes recognized by CTL. These peptides usually contain certain amino acids at some positions called anchor positions that define sequence patterns or motifs [22]. By using these HLA binding motifs, several CTL epitopes from HCV antigens have been synthesized and tested [13,14,23–25]. These peptides have first been tested in binding assays and binder peptides used to stimulate PBMC from HCV patients. However, not all experimentally identified peptides as CTL epitopes fulfil these motifs [17], indicating that other peptides without the common binding motif may exist and have not yet been identified. Identification of CTL epitopes in the HCV

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proteins is of great importance, because they may be useful for vaccine development, treat chronic infection or monitor anti-HCV CTL immune responses.

In order to identify new CTL epitopes from HCV structural proteins we synthesized 9-mer peptides predicted as binders to HLA-A2 by an algorithm, and overlapping 14-15-mer peptides spanning almost the entire structural region. We show below that binding to HLA-A2, one of the more common HLA molecules, can be detected using both types of peptides. Moreover, in vitro stimulation of cells from HCV infected patients allowed us to identify CTL activity against peptide E2(614-622), not previously characterized as CTL epitope. These findings are discussed in more detail below.

2. Materials and methods

2.1. Synthetic peptides

Peptides were prepared manually as described [26]. Purity of the peptides was always above 80% as judged by HPLC. Nine-mer peptides were selected using an algorithm developed in our laboratory based on published data [22]. This algorithm searches for peptides containing the HLA-A2 binding motif.

2.2. Cells

Cell lines T2 (gift of Dr Jay A. Berzofsky (NIH, Bethesda, USA)), JY (HLA-A2.1, B7, Cw7), kindly gift of Dr Andreas Cerny (Bern, Switzerland), and BE (HLA-A24/26, B27/44), generated in our laboratory, were maintained in complete medium (CM; RPMI containing 10% foetal bovine serum (FBS), 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol).

2.3. HCV patients

Anti-HCV immune response was studied in 25 patients with chronic hepatitis C (13 HLA-$A2^+$ and 12 HLA-$A2^-$). They had anti HCV-specific antibodies and were HCV RNA positive by PCR. HLA-A2 typing was done by using the anti-HLA-A2 BB7.2 monoclonal antibody (ATCC, Manassas, VA). Twelve healthy seronegative HLA-$A2^+$ individuals were included as controls. This study was approved by the institution's human research committee and informed consent was obtained from the individuals.

2.4. Binding assays

Peptide binding to HLA-A2 molecules was measured using the T2 mutant cell line according to a protocol previously described [27]. Briefly, T2 cells were incubated overnight in 96-well plates with peptide (100 μM). Next day cells were washed and incubated with anti-HLA-A2 BB7.2 antibody and FITC-labelled goat anti mouse Ig. HLA-A2 expression was measured by flow cytometry, and quantified as fluorescence index (FI) according to the formula: FI = (mean fluorescence with peptide - mean fluorescence without peptide)/ mean fluorescence without peptide.

2.5. CTL lines generation

CTL lines were generated as previously described [27]. PBMC (2×10^6 cells) were stimulated in 1 ml in 24-well plates with 4×10^6 autologous cells pulsed for 3 h with 10 μM peptide or peptide mixtures in CM. After 3 days, CM with IL-2 (10 U/ml) was added to each well and the cells were further expanded on day six with IL-2 containing medium. T cells (2×10^6/well) were stimulated every ten days with 4×10^6 peptide pulsed irradiated (3000 rads) autologous PBMC and expanded in IL-2 containing medium.

2.6. Cytotoxicity assay

CTL activity was measured using a 4-h assay with ^{51}Cr-labeled target cells. Autologous Concanavaline A-stimulated blasts or EBV-transformed B cell lines JY and BE were used as targets.

2.7. Intracellular cytokine detection

T cells (10^5) were incubated in 96-well plates for 5 h at 37°C with JY cells (10^4) with or without peptide, in the presence of 10 U/ml IL-2 and monensin (GolgiStop®; Pharmingen, San Diego, CA) according to manufacturer's instructions. After this period, cells were washed and stained with PE-labeled anti-CD8 antibodies. Cells were fixed and permeabilized with Cytofix/Cytoperm® (Pharmingen), stained with FITC-labeled anti-IFN-γ antibodies and analyzed by flow cytometry on a FACScan.

2.8. ELISPOT assay

Analysis of anti-peptide immune response in the different groups of individuals was carried out by using an IFN-γ-based ELISPOT assay (Mabtech, Sweden) according to manufacturer's instructions. Briefly, 96-well nitrocellulose-backed plates (MultiScreen-HA; Millipore, Bedford, MA) were coated with capture antibody overnight at 4°C. Next day, 10^5 $CD8^+$ purified cells and 5×10^4 mitomycin C-treated T2 cells were cultured in triplicate wells. Peptide was added in some wells at 50 μM. After 40 h of culture at 37°C and 5% CO_2, wells were washed and incubated with detection antibody for 3 h at room temperature. After 1.5 h of incubation with Streptavidin-AP, plates were washed and colour reaction was developed by adding BCIP/NBT substrate solution (BioRad, Hercules, CA). Reaction was stopped after 30 min with distilled water, and spot forming cells were counted. Responses were considered positive when the number of spots per well were at least twice that in control wells.

2.9. HCV sequences

Sequences used to synthesize peptide variants of the CTL epitope studied were collected from the literature. Sequences belonged to genotypes 1a, 1b, 2a and 2b (7, 15, 2 and 2 sequences, respectively). Gene Bank accession numbers for these sequences were the following: M62321, M67463, M62382, X53134, D16699, X53133, D10664, D90208, D11168/D01171, D11355/D01172, M58335, M84754, D10750, D13558/D01217, L02836, D90077/M57581, X61591, X61593, X61592, X61594, X61595, M86766, D00944, D10075/6, D01221 and D10077/8.

3. Results

3.1. Binding of peptides from HCV structural proteins to HLA-A2 molecules

In order to identify HLA-A2 binding peptides from HCV structural proteins that could be potential CTL epitopes, we used an algorithm that predicts peptide binding and synthesized thirty two 9-mer peptides. Binding of these 9-mer peptides to HLA-A2 was measured using T2 cells. Four peptides from core, 2 from E1 and 3 from E2 showed some degree of binding to HLA-A2 (Table 1). To study if other peptides not predicted by algorithms could bind to HLA-A2, we measured binding using 117 overlapping peptides from HCV proteins: 39 fourteen-mer peptides encompassing core protein, 36 fifteen-mer peptides encom-

passing E1 and 42 fifteen-mer peptides encompassing 220 N-terminal amino acids from E2. This allowed us to use peptides spanning all the possible 8-10-mer peptides encompassed by HCV structural proteins. Three peptides from core, three form E1 and five from E2 proteins showed positive results in binding assays, although they had lower binding ability (as expressed by Fluorescence Index) than 9-mer peptides (Table 1).

3.2. Growth of CTL lines from an HCV$^+$ patient PBMC by stimulation with binder peptides

CTL epitope character of the best binder peptides was tested by in vitro stimulation of PBMC from an HCV$^+$ HLA-A2$^+$ patient with these peptides. Two peptide pools were used: pool #1, containing peptides c(132-140), c(168-176), c(178-191), E1(285-293) and E2(614-622), and pool #2, containing peptides E1(317-331), E1(322-336), E2(424-438) and E2(684-692). After three stimulation cycles, cells stimulated with peptide pool #1 had specific lytic activity against peptide pulsed target cells, whereas cells stimulated with pool #2 did not show any activity (Fig. 1A). When CTL specific for pool #1 where tested against target cells pulsed with individual peptides, only those pulsed with E2(614-622) were lysed. This shows that CTL activity is due to E2(614-622) only (Fig. 1B). In vitro priming of naive CTL was discarded because a similar protocol did not yield any activity when used with PBMC from four HLA-A2$^+$ HCV seronegative donors (Fig. 1C).

Experiments using HLA-A2$^+$ and HLA-A2$^-$ target cells showed that HLA-A2 was the molecule presenting E2(614-622) to CTL (Fig. 2A). The phenotype of CTL was assessed by flow cytometry using an intracellular cytokine detection assay, showing that CD8$^+$ cells are the main population producing IFN-γ after peptide stimulation. (Fig. 2B,C).

To study whether responses against peptides other than E2(614-622) may co-exist but were masked by competition for HLA-A2, responses against individual peptides were tested by using an IFN-γ-based ELISPOT assay. These experiments showed that E2(614-622) was recognized by the highest number of cells (Fig. 3), confirming its immunodominant role in the peptide pool. Moreover, weaker responses against c(132-140) and E1(285-293) were also detected.

3.3. Recognition of peptide E2(614-622) by patients with chronic hepatitis C

After characterizing the presence of CTL specific for E2(614-622) in a particular patient, it was important to know the prevalence of the response to this peptide in chronic HCV infection. Thus, we studied this response in a group of 13 HLA-A2$^+$ patients with chronic hepatitis C by using the IFN-γ-based ELISPOT assay, that would also provide data on precursor cell frequency. When CD8$^+$ cells from HLA-A2$^+$ patients were stimulated with peptide E2(614-622), specific responses were detected in 30% of them (Fig. 4A). We studied a group of HLA-A2$^-$ patients with chronic hepatitis C and a group of healthy HLA-A2$^+$ seronegative individuals as controls. In none of these groups we obtained a positive response (Fig. 4B,C), showing that recognition of epitope E2(614-622) is specific of HLA-A2$^+$ subjects, and that response to this peptide is induced by viral infection.

3.4. HLA binding and CTL recognition of sequences from region E2(614-622) present in other viral isolates

A characteristic feature of HCV is the great variability present among the different viral isolates [28], particularly in envelope proteins. This variability has been interpreted by several authors as a viral strategy to avoid immune responses [29–32]. We analyzed region E2(614-622) in 26 viral isolates belonging to genotypes 1a, 1b, 2a and 2b (Table 2). As it can be seen, only changes at position 622 (amino acid 9 of the peptide) were found. Most of the sequences (18 out of 26) had V at position 9, as the peptide used to stimulate the CTL. Four other amino acids were found at this position, I, L, F and A, in 4, 2, 1 and 1 viral isolates, respectively. Position 9 has been described as one of the important anchor positions for peptide binding to HLA-A2. Thus, these changes may affect peptide binding and concomitantly CTL recognition. In order to study these possibilities, we synthesized peptides containing these substitutions and measured their ability to bind to HLA-A2. Peptides E2(614-622) (with V at position 9) and E2(614-622)I have the same degree of binding, E2(614-622)L and E2(614-622)A have slightly less binding ability, whereas E2(614-622)F needs higher peptide concentrations to reach the same levels of binding (Fig. 5A). We also tested the peptides in a CTL assay, to study the effect of these mutations on CTL recognition. This experiment showed that peptides with V, I, L or A at position 9 were almost equally recognized by the CTL line thus induced (Fig. 5B). However, peptide E2(614-622)F was poorly recognized, showing a low activity only at the highest peptide concentration. These results were confirmed by independent experiments done with a different CTL line raised also against E2(614-622)V (data not shown).

4. Discussion

CTL are important effector cells in the control of many viral diseases [33,34]. In the case of HCV infection, many individuals become chronically infected despite the presence of CTL. Although this may argue against the role of CTL in the control of HCV infection, recent reports show the importance of CTL in the final outcome of the infection [18,35], in the protection of individuals in contact with HCV patients [19,20] or in patients clearing HCV after α-IFN treatment [36]. Thus, strategies aimed to stimulate CTL responses would be of great importance. For this reason, identification of relevant CTL epitopes from

Table 1
Binding to HLA-A2 molecules of HCV structural region derived peptides[a]

Peptide	Sequence	Binding	Peptide	Sequence	Binding	Peptide	Sequence	Binding
C(29-37)	QIVGGVYLL	−	E1(245-253)	TLAAKDASI	−	E2(398-406)	GLTSLFTPG	−
C(36-44)	LLPRRGPRL	−	E1(263-271)	DLLVGAAAF	−	E2(432-440)	SLQTGFLAA	−
C(38-46)	PRRGPRLGV	−	E1(264-272)	LLVGAAAFC	−	E2(614-622)	RLWHYPCTV	++
C(97-105)	LLSPRGSRP	−	E1(279-287)	DLCGSVFLV	+	E2(621-629)	TVNFTIFKV	−
C(118-126)	NLGKVIDTL	+	E1(285-293)	FLVSQLFTF	+++	E2(625-633)	TIFKVRMYV	−
C(132-140)	DLMGYIPLV	+++	E1(336-344)	QLLRIPQAV	−	E2(628-636)	KVRMYVGGV	−
C(138-146)	PLVGAPLGG	−	E1(337-345)	LLRIPQAVV	−	E2(684-692)	ALSTGLIHI	+++
C(143-151)	PLGGAARAL	−	E1(355-363)	VLAGLAYYS	−	E2(701-709)	YLYGVGSAV	−
C(150-158)	ALAHGVRVL	−	E1(363-371)	SMVGNWAKV	−	E2(720-728)	LLLFLLLAD	−
C(168-176)	NLPGCSFSI	++	E1(365-373)	VGNWAKVLV	−	E2(723-731)	FLLLADARV	+
C(180-188)	ALLSCLTIP	+	E1(373-381)	VVMLLFAGV	−			
C(1-14)	MSTIPKPQRKTKRN	−	E1(192-206)	YEVRNVSGIYHVTND	−	E2(384-398)	EPYTTGGTHGRAAHG	−
C(6-19)	KPQRKTKRNTNRRP	−	E1(197-211)	VSGIYHVTNDCSNSS	−	E2(389-403)	GGTHGRAAHGLTSLF	−
C(11-14)	TKRNTNRRPQDVKF	−	E1(202-216)	HVTNDCSNSSIVYET	−	E2(394-408)	RAAHGLTSLFTPGPA	−
C(15-28)	TNRRPQDVKFPGGG	−	E1(207-221)	CSNSSIVYETADMIM	−	E2(399-413)	LTSLFTPGPAQKIQL	−
C(20-33)	QDVKFPGGGQIVGG	−	E1(212-226)	IVYETADMIMHTPGC	−	E2(404-418)	TPGPAQKIQLVNTNG	−
C(25-38)	PGGGQIVGGVYLLP	−	E1(217-231)	ADMIMHTPGCVPCVR	−	E2(409-423)	QKIQLVNTNGSWHIN	−
C(29-42)	QIVGGVYLLPRRGP	−	E1(222-236)	HTPGCVPCVREGNSS	−	E2(414-428)	VNTNGSWHINRTALN	−
C(34-47)	VYLLPRRGPRLGVR	+	E1(227-241)	VPCVREGNSSRCWVA	−	E2(419-433)	SWHINRTALNCNDSL	−
C(39-52)	RRGPRLGVRATRKT	−	E1(232-246)	EGNSSRCWVALTPTL	−	E2(424-438)	RTALNCNDSLQTGFL	++
C(43-56)	RLGVRATRKTSERS	−	E1(237-251)	RCWVALTPTLAAKDA	−	E2(429-443)	CNDSLQTGFLAALFY	+
C(48-61)	ATRKTSERSQPRGR	−	E1(242-256)	LTPTLAAKDASIPTA	−	E2(434-448)	QTGFLAALFYTHRFN	−
C(53-66)	SERSQPRGRRQPIP	−	E1(247-261)	AAKDASIPTATIRRH	−	E2(439-453)	AALFYTHRFNASGCS	−
C(57-70)	QPRGRRQPIPKVRR	−	E1(252-266)	SIPTATIRRHVDLLV	−	E2(444-458)	THRFNASGCSERMAS	−
C(62-75)	RQPIPKVRRPEGRT	−	E1(257-271)	TIRRHVDLLVGAAAF	−	E2(449-463)	ASGCSERMASCRPID	+
C(67-80)	KVRRPEGRTWAQPG	−	E1(262-276)	VDLLVGAAAFCSAMY	−	E2(454-468)	ERMASCRPIDQFDQG	−
C(71-84)	PEGRTWAQPGYPWP	−	E1(267-281)	GAAAFCSAMYVGDLC	−	E2(459-473)	CRPIDQFDQGWGPIT	−
C(76-89)	WAQPGYPWPLYGNE	−	E1(272-286)	CSAMYVGDLCGSVFL	+	E2(464-478)	QFDQGWGPITYNESH	−
C(81-94)	YPWPLYGNEGCGWA	−	E1(277-291)	VGDLCGSVFLVSQLF	−	E2(469-483)	WGPITYNESHGLDQR	−
C(85-98)	LYGNEGCGWAGWLL	−	E1(282-296)	GSVFLVSQLFTFSPR	−	E2(474-488)	YNESHGLDQRPYCWH	+
C(90-103)	GCGWAGWLLSPRGS	−	E1(287-301)	VSQLFTFSPRRHQTV	−	E2(479-493)	GLDQRPYCWHYAPQP	−
C(95-108)	GWLLSPRGSRPSWG	−	E1(292-306)	TFSPRRHQTVQDCNC	−	E2(484-498)	PYCWHYAPQPCGIVP	−
C(99-112)	SPRGSRPSWGPTDP	−	E1(297-311)	RHQTVQDCNCSIYPG	−	E2(489-503)	YAPQPCGIVPALQVC	−
C(104-117)	RPSWGPTDPRRRSR	−	E1(302-316)	QDCNCSIYPGHVSGH	−	E2(494-508)	CGIVPALQVCGPVYC	−
C(109-122)	PTDPRRRSRNLGKV	−	E1(307-321)	SIYPGHVSGHRMAWD	−	E2(499-513)	ALQVCGPVYCFTPSP	+
C(113-126)	RRRSRNLGKVIDTL	−	E1(312-326)	HVSGHRMAWDMMMNW	−	E2(504-518)	GPVYCFTPSPVVVGT	−
C(118-131)	NLGKVIDTLTCGFA	−	E1(317-331)	RMAWDMMMNWSPTAA	++	E2(509-523)	FTPSPVVVGTTDRFG	−
C(123-136)	IDTLTCGFADLMGY	−	E1(322-336)	MMMNWSPTAALVVSQ	++	E2(514-528)	VVVGTTDRFGAPTYR	−
C(127-140)	TCGFADLMGYIPLV	−	E1(327-341)	SPTAALVVSQLLRIP	−	E2(519-533)	TDRFGAPTYRWGENE	−
C(132-145)	DLMGYIPLVGAPLG	++	E1(332-346)	LVVSQLLRIPQAVVD	−	E2(524-538)	APTYRWGENETDVLL	−
C(137-150)	IPLVGAPLGGAARA	−	E1(337-351)	LLRIPQAVVDMVAGA	−	E2(529-543)	WGENETDVLLLNNTR	−
C(141-154)	GAPLGGAARALAHG	−	E1(342-356)	QAVVDMVAGAHWGVL	−	E2(534-548)	TDVLLLNNTRPPRGN	−
C(146-159)	GAARALAHGVRVLE	−	E1(347-361)	MVAGAHWGVLAGLAY	−	E2(539-553)	LNNTRPPRGNWFGCT	−
C(151-164)	LAHGVRVLEDGVNY	−	E1(352-366)	HWGVLAGLAYYSMVG	−	E2(544-558)	PPRGNWFGCTWMNST	−

Table 1 (*continued*)

Peptide	Sequence	Binding	Peptide	Sequence	Binding	Peptide	Sequence	Binding
C(155-168)	VRVLEDGVNYATGN	−	E1(357-371)	AGLAYYSMVGNWAKV	−	E2(549-563)	WFGCTWMNSTGFTKT	−
C(160-173)	DGVNYATGNLPGCS	−	E1(362-376)	YSMVGNWAKVLVVML	−	E2(554-568)	WMNSTGFTKTCGGPP	−
C(165-178)	ATGNLPGCSFSIFL	−	E1(367-381)	NWAKVLVVMLLFAGV	−	E2(559-573)	GFTKTCGGPPCNIGG	−
C(169-182)	LPGCSFSIFLLALL	−				E2(564-578)	CGGPPCNIGGVGNNT	−
C(173-186)	SFSIFLLALLSCLT	−				E2(569-583)	CNIGGVGNNTLICPT	−
C(178-191)	LLALLSCLTVPASA	+				E2(574-588)	VGNNTLICPTDCFRK	−
						E2(579-593)	LICPTDCFRKHPEAT	−
						E2(584-598)	DCFRKHPEATYTKCG	−
						E2(589-603)	HPEATYTKCGSGPWL	−

[a] Binding ability of peptides is expressed as Fluorescence Index (FI = (mean fluorescence with peptide - mean fluorescence without peptide)/mean fluorescence without peptide). Results were scored as: (−) FI < 0.5, (+) 0.5 < FI < 1, (+ +) 1 < FI < 2, and (+++) FI > 2. Overlapping 14-15-mer peptides contain an extra Ala at the C terminus added for synthesis convenience.

constant regions of HCV proteins is of paramount importance. CTL epitopes must bind to HLA molecules, thus, as a first approach, we identified peptides able to bind to HLA-A2. Two strategies have been used to identify binder peptides, synthesis of 9-mer peptides having a potential HLA-A2 binding motif and synthesis of overlapping 14-15-mer peptides. Both methods have proven useful for the detection of binder peptides, but higher binding ability was usually found for 9-mer peptides. This is probably due to their ability to bind directly to HLA molecules. Most of the 14-15-mer binder peptides contained the binding motif, although some of them, like E2(449-463) or E2(474-488) did not contain any putative binding motif. Thus, we favour the use of binding algorithms to detect binder peptides, but

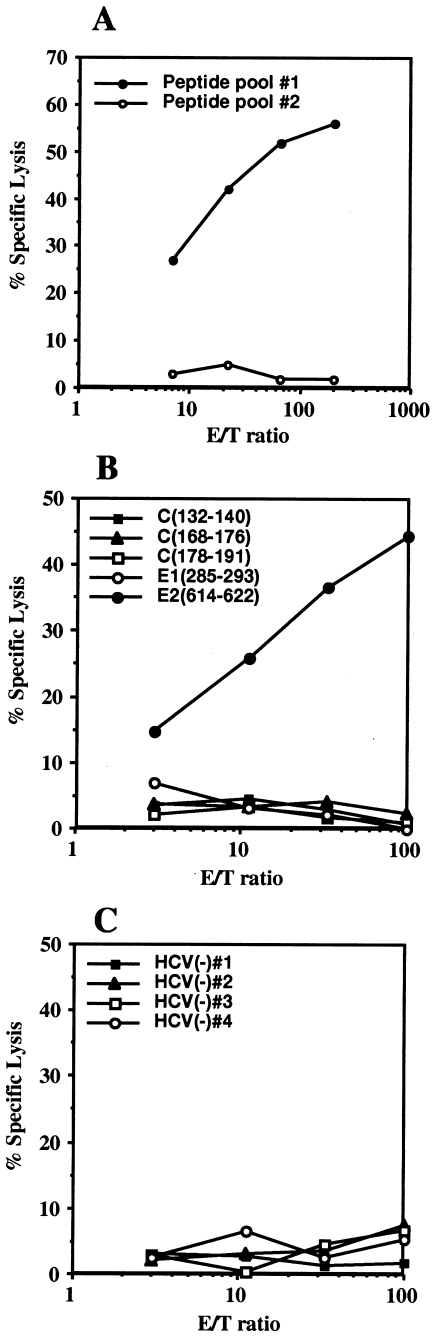

Fig. 1. (A) CTL activity of cells from an HLA-A2$^+$ HCV patient stimulated with HLA-A2 binding peptides: pool #1 (peptides c(132-140), c(168-176), c(178-191), E1(285-293) and E2(614-622)) and pool #2 (peptides E1(317-331), E1(322-336), E2(424-438) and E2(684-692)). Results shown represent the difference in specific lysis between peptide pulsed and unpulsed target cells. (B) Characterization of peptides within pool #1 recognized by CTL. (C) CTL activity against E2(614-622) obtained in four HCV HLA-A2$^+$ seronegative donors.

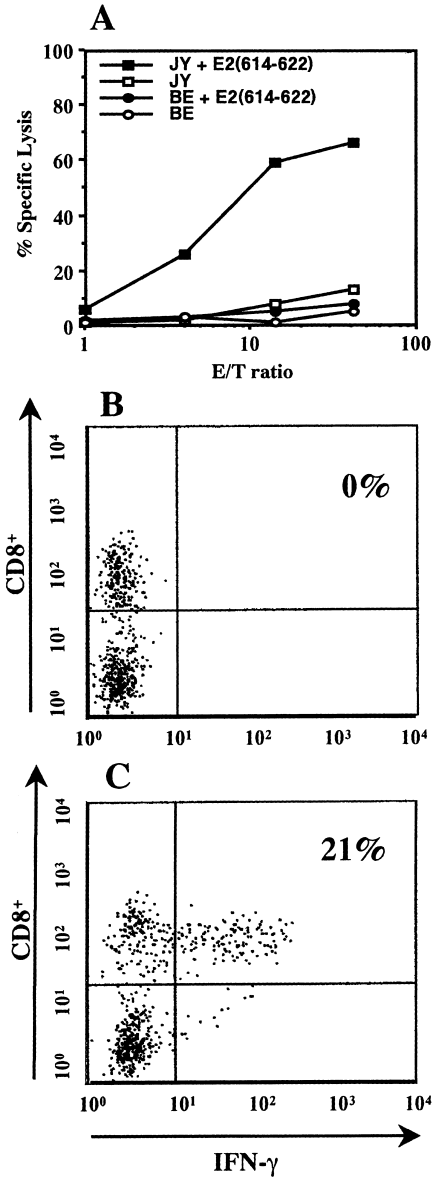

Fig. 2. (A) Characterization of the HLA molecule presenting peptide E2(614-622) to CTL. JY cells (HLA-A2$^+$) and BE cells (HLA-A2$^-$) were used as targets. (B-C) Phenotype of CTL recognizing peptide E2(614-622). T cells were incubated with HLA-A2$^+$ JY cells in the absence (B) or in the presence (C) of E2(614-622), and stained with PE-labeled anti-CD8 and FITC-labeled anti-γ-IFN antibodies to analyze the phenotype of peptide-specific T cells by flow cytometry.

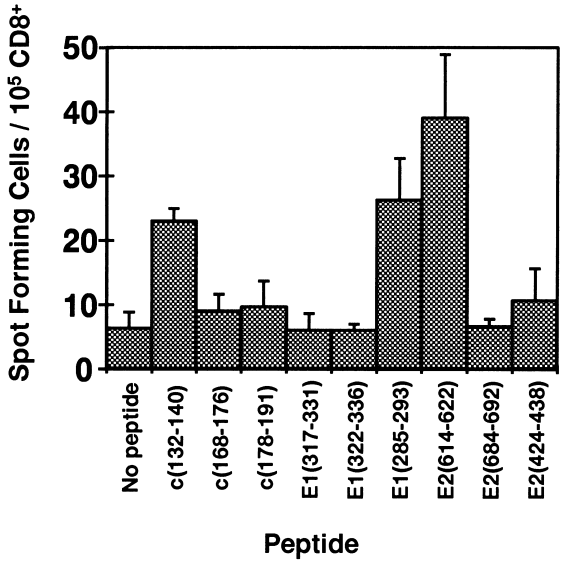

Fig. 3. Recognition of HLA-A2 binder peptides by $CD8^+$ purified cells by ELISPOT. $CD8^+$ cells were incubated with T2 cells with or without peptides (50 μM) in plates coated with anti IFN-γ antibodies. Then, spots were detected as described in Materials and Methods and counted.

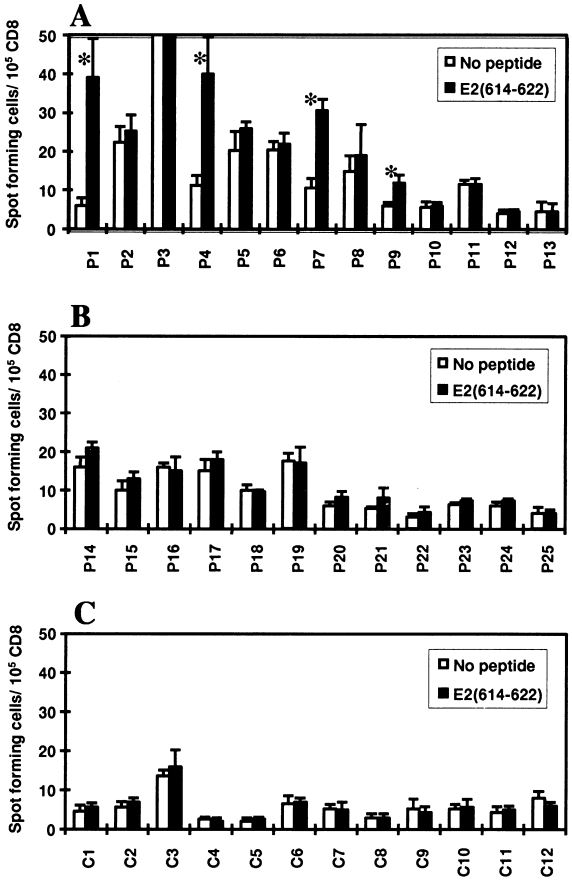

Fig. 4. Recognition of E2(614-622) peptide by $CD8^+$ cells by ELISPOT. (A) $HLA\text{-}A2^+$ patients with chronic hepatitis C, (B) $HLA\text{-}A2^-$ patients with chronic hepatitis C and (C) $HLA\text{-}A2^+$ healthy individuals. Patients labelled with (*) represent positive responses.

Table 2
Sequence of peptides from region E2(614-622) belonging to different viral isolates

Peptide sequence[a]	Number of isolates
RLWHYPCT**V**	18
RLWHYPCT**I**	4
RLWHYPCT**L**	2
RLWHYPCT**A**	1
RLWHYPCT**F**	1

[a] Variable residues are shown in bold.

this strategy could be complemented by using also overlapping 14-15-mers.

In order to identify new CTL epitopes, peptides with the highest ability to bind to HLA-A2 were used to stimulate PBMC from a patient chronically infected by HCV. These experiments led to the growth of CTL lines specific for E2(614-622). This peptide was recognized by $CD8^+$ CTL in an HLA-A2 restricted manner. When peptides were tested individually using $CD8^+$ cells from the same patient by using an ELISPOT assay, the strongest IFN-γ production was induced by peptide E2(614-122). Although competition for HLA-A2 may prevent detection of responses against other peptides in chromium release assays, the low number of precursor cells seems to be the main reason to explain this phenomenon. Although we have only detected strong responses against E2(614-622), one can not rule out the possibility that some of the binder peptides used in the present study, and not characterized as CTL epitopes, might be able to stimulate CTL in other patients.

Analysis of the prevalence of the response to this peptide in a wider group of patients with chronic hepatitis C showed that 30% of them recognized the peptide. The number of E2(614-622)-specific precursor cells in those patients with positive responses was about 10–30/10^5 $CD8^+$ cells. In these cases, persistence of viral replication in the presence of CTL might be due to the low number of cells. Detection of CTL responses against E2(614-622) by chromium release assays required at least three in vitro stimulation cycles, and reactivity against c(132-140) and E1(285-293) could be detected only by ELISPOT, reinforcing the idea that their number is indeed very low.

Sequence variability is a characteristic of HCV that may have important implications for the development of a protective immune response. Mutations in some immunogenic regions have been described, allowing the emergence of escape mutants both at the B- and T-cell level [29–32,37]. The use of epitopes belonging to immunologically conserved regions is thus important [24]. Variability in region E2(614-622) was localized at position 622, position 9 of the peptide sequence. Because this position plays the role of an anchor to HLA-A2, modification of this residue may affect peptide-HLA binding and CTL recognition. HLA-A2 usually allows the binding of peptides with aliphatic residues at position 9 [22]. In this case, most of

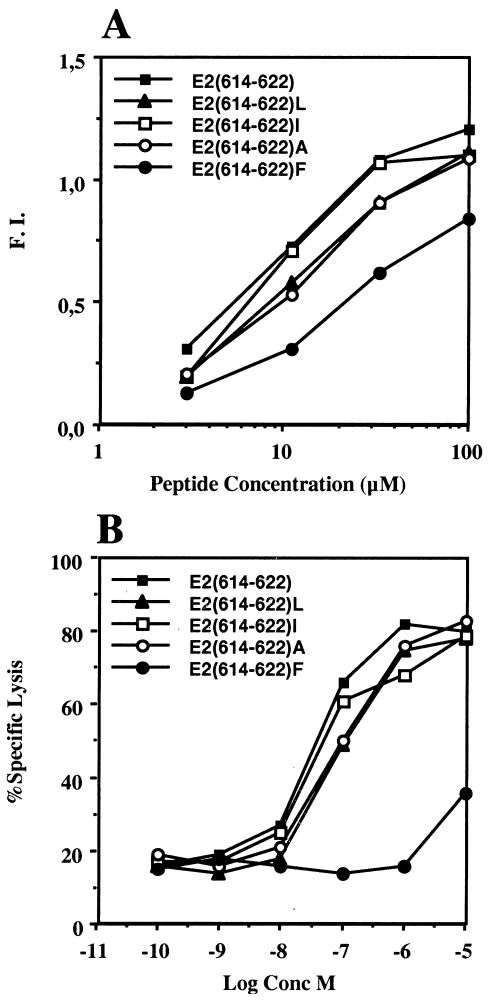

Fig. 5. (A) Binding to HLA-A2 molecules of E2(614-622) derived peptides with substitutions at position 622. T2 cells were incubated with different peptide concentrations and HLA-A2 expression was measured by flow cytometry. Results are expressed as fluorescence index. (B) Recognition of E2(614-622) derived peptides by CTL specific for E2(614-622). Chromium-labeled JY cells incubated with different peptide concentrations were used as targets. An effector/target ratio of 10:1 was used.

the mutations found at this position presented amino acids of this type. Thus, only substitution by F at position 622 had an important effect in binding and, as a consequence, in CTL recognition. In the other viral isolates, although they present mutations at this position, these mutations did not affect binding to HLA-A2 and CTL recognition. This means that most of the mutations in this position could be considered immunologically silent. As F is only present in 1 out of 26 sequences studied, from an immunological point of view, E2(614-622) could be considered as a conserved epitope.

In summary, peptide E2(614-622) has been characterized as a new CTL epitope in HLA-A2$^+$ chronically infected patients. This peptide belongs to a region which is very well preserved with respect to CTL recognition, suggesting the potential use of this epitope in strategies aiming at enhancing anti-HCV cell immunity.

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References

[1] Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989;244:359–362.

[2] Dienstag JL. Non-A, non-B hepatitis. I. Recognition, epidemiology, and clinical features. Gastroenterology 1983;85:439–462.

[3] Van der Poel CL, Cuypers HT, Reesink HW, Weiner AJ, Quan S, Di Nello R, et al. Confirmation of hepatitis C virus infection by new four-antigen recombinant immunoblot assay. Lancet 1991;337:317–319.

[4] Botarelli P, Brunetto MR, Minutello MA, Calvo P, Unutmaz D, Weiner AJ, et al. T-lymphocyte response to hepatitis C virus in different clinical courses of infection. Gastroenterology 1993;104:580–587.

[5] Minutello MA, Pileri P, Unutmaz D, Censini S, Kuo G, Houghton M, et al. Compartmentalization of T lymphocytes to the site of disease: intrahepatic CD4$^+$ T cells specific for the protein NS4 of hepatitis C virus in patients with chronic hepatitis C. J Exp Med 1993;178:17–25.

[6] Diepolder HM, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, Jung MC, et al. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 1995;346:1006–1007.

[7] Hoffmann RM, Diepolder HM, Zachoval R, Zwiebel FM, Jung MC, Scholz S, et al. Mapping of immunodominant CD4$^+$ T lymphocyte epitopes of hepatitis C virus antigens and their relevance during the course of chronic infection. Hepatology 1995;21:632–638.

[8] Iwata K, Wakita T, Okumura A, Yoshioka K, Takayanagi M, Wands JR, et al. Interferon gamma production by peripheral blood lymphocytes to hepatitis C virus core protein in chronic hepatitis C infection. Hepatology 1995;22:1057–1064.

[9] Sarobe P, Jauregui JI, Lasarte JJ, Garcia N, Civeira MP, Borras-Cuesta F, et al. Production of interleukin-2 in response to synthetic peptides from hepatitis C virus E1 protein in patients with chronic hepatitis C: relationship with the response to interferon treatment. J Hepatol 1996;25:1–9.

[10] Lasarte JJ, Garcia Granero M, Lopez A, Casares N, Garcia N, Civeira MP, et al. Cellular immunity to hepatitis C virus core protein and the response to interferon in patients with chronic hepatitis C. Hepatology 1998;28:815–822.

[11] Koziel MJ, Dudley D, Wong JT, Dienstag J, Houghton M, Ralston R, et al. Intrahepatic cytotoxic T lymphocytes specific for hepatitis C virus in persons with chronic hepatitis. J Immunol 1992;149:3339–3344.

[12] Koziel MJ, Dudley D, Afdhal N, Choo QL, Houghton M, Ralston R, et al. Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes recognize epitopes in the core and envelope proteins of HCV. J Virol 1993;67:7522–75232.

[13] Battegay M, Fikes J, Di Bisceglie AJ, Wentworth PA, Sette A, Celis E, et al. Patients with chronic hepatitis C have circulating cytotoxic T cells which recognize hepatitis C virus-encoded peptides binding to HLA-A2.1 molecules. J Virol 1995;69:2462–2470.

[14] Cerny A, McHutchison JG, Pasquinelli C, Brown ME, Brothers MA, Grabscheid B, et al. Cytotoxic T lymphocyte response to hepatitis C

virus-derived peptides containing the HLA A2.1 binding motif. J Clin Invest 1995;95:521–530.

[15] Shirai M, Okada H, Nishioka M, Akatsuka T, Wychowski C, Houghten R, et al. An epitope in hepatitis C virus core region recognized by cytotoxic T cells in mice and humans. J Virol 1994;68:3334–3342.

[16] Kita H, Hiroishi K, Moriyama T, Okamoto H, Kaneko T, Ohnishi S, et al. A minimal and optimal cytotoxic T cell epitope within hepatitis C virus nucleoprotein. J Gen Virol 1995;76:3189–3193.

[17] Kurokohchi K, Akatsuka T, Pendleton CD, Takamizawa A, Nishioka M, Battegay M, et al. Use of recombinant protein to identify a motif-negative human cytotoxic T-cell epitope presented by HLA-A2 in the hepatitis C virus NS3 region. J Virol 1996;70:232–240.

[18] Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY, et al. Analysis of a successful immune response against hepatitis C virus. Immunity 1999;10:439–449.

[19] Koziel MJ, Wong DK, Dudley D, Houghton M, Walker BD, Hepatitis C. virus-specific cytolytic T lymphocyte and T helper cell responses in seronegative persons. J Infect Dis 1997;176:859–866.

[20] Scognamiglio P, Accapezzato D, Casciaro MA, Cacciani A, Artini M, Bruno G, et al. Presence of effector $CD8^+$ T cells in hepatitis C virus-exposed healthy seronegative donors. J Immunol 1999;162:6681–6689.

[21] Townsend A, Bodmer H. Antigen recognition by class I-restricted T lymphocytes. Annu Rev Immunol 1989;7:601–624.

[22] Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 1991;351:290–296.

[23] Shirai M, Arichi T, Nishioka M, Nomura T, Ikeda K, Kawanishi K, et al. CTL responses of HLA-A2.1-transgenic mice specific for hepatitis C viral peptides predict epitopes for CTL of humans carrying HLA-A2.1. J Immunol 1995;154:2733–2742.

[24] Wentworth PA, Sette A, Celis E, Sidney J, Southwood S, Crimi C, et al. Identification of A2-restricted hepatitis C virus-specific cytotoxic T lymphocyte epitopes from conserved regions of the viral genome. Int Immunol 1996;8:651–659.

[25] Chang KM, Gruener NH, Southwood S, Sidney J, Pape GR, Chisari FV, et al. Identification of HLA-A3 and -B7-restricted CTL response to hepatitis C virus in patients with acute and chronic hepatitis C. J Immunol 1999;162:1156–1164.

[26] Borras Cuesta F, Golvano J, Sarobe P, Lasarte JJ, Prieto I, Szabo A, et al. Insights on the amino acid side-chain interactions of a synthetic T-cell determinant. Biologicals 1991;19:187–190.

[27] Sarobe P, Pendleton CD, Akatsuka T, Lau D, Engelhard VH, Feinstone SM, et al. 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. J Clin Invest 1998;102:1239–1248.

[28] Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, et al. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol 1993;74:2391–2399.

[29] Chang KM, Rehermann B, McHutchison JG, Pasquinelli C, Southwood S, Sette A, et al. Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. J Clin Invest 1997;100:2376–2385.

[30] Kaneko T, Moriyama T, Udaka K, Hiroishi K, Kita H, Okamoto H, et al. Impaired induction of cytotoxic T lymphocytes by antagonism of a weak agonist borne by a variant hepatitis C virus epitope. Eur J Immunol 1997;27:1782–1787.

[31] Tsai SL, Chen YM, Chen MH, Huang CY, Sheen IS, Yeh CT, Hepatitis C, et al. virus variants circumventing cytotoxic T lymphocyte activity as a mechanism of chronicity. Gastroenterology 1998;115:954–965.

[32] Frasca L, Del Porto P, Tuosto L, Marinari B, Scotta C, Carbonari M, et al. Hypervariable region 1 variants act as TCR antagonists for hepatitis C virus-specific CD4 + T cells. J Immunol 1999;163:650–658.

[33] Taylor PM, Askonas BA. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective in vivo. Immunology 1986;58:417–420.

[34] Schulz M, Zinkernagel RM, Hengartner H. Peptide-induced antiviral protection by cytotoxic T cells. Proc Natl Acad Sci USA 1991;88:991–993.

[35] Takaki A, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, et al. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat Med 2000;6:578–582.

[36] Lohr HF, Schmitz D, Arenz M, Weyer S, Gerken G, Meyer zum Buschenfelde KH. The viral clearance in interferon-treated chronic hepatitis C is associated with increased cytotoxic T cell frequencies. J Hepatol 1999;31:407–415.

[37] Weiner AJ, Geysen HM, Christopherson C, Hall JE, Mason TJ, Saracco G, et al. Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. Proc Natl Acad Sci USA 1992;89:3468–3472.