Induction of Cytotoxic T-Cell Response Against Hepatitis C Virus Structural Antigens Using a Defective Recombinant Adenovirus

Oscar Bruña-Romero,[1] Juan José Lasarte,[1] Gavin Wilkinson,[2] Ken Grace,[3] Berwyn Clarke,[3] Francisco Borrás-Cuesta,[1] and Jesús Prieto[1]

A replication-defective recombinant adenovirus (RAd), RAdCMV-CE1, containing core and E1 genes of hepatitis C virus (HCV) was constructed. RAdCMV-CE1 was able to express core and E1 proteins both in mice and human cells. Immunization of BALB/c mice with RAdCMV-CE1 induced a specific cytotoxic T-cell response against the two HCV proteins. This response was characterized using a panel of 60 synthetic 14- or 15-mer overlapping peptides (10 amino-acid overlap) spanning the entire sequence of these proteins. Five main epitopes were found in the core protein, four of which had been previously described either in mice or humans. One single novel epitope was found in E1. Fine mapping of this E1 determinant, showed that octamer GHRMAWDM is the minimal epitope recognized by cytotoxic T lymphocytes (CTL). The cytotoxic T-cell response was *H-2*^{d} restricted, lasted for at least 100 days, and was mediated by T cells with the classic CD4$^-$ CD8$^+$ phenotype. This work demonstrates that replication-defective recombinant adenoviruses can efficiently express HCV proteins and are able to induce an *in vivo* cytotoxic T-cell response against a diversity of epitopes from HCV antigens. These vectors should be taken into consideration in the design of vaccines and also as a means to stimulate specific T-cell responses in chronic HCV carriers. (Hepatology 1997;25: 470-477.)

Hepatitis C virus (HCV) is one of the major agents of chronic hepatitis and liver diseases worldwide.[1] Infection with HCV leads to chronic hepatitis in about 80% of cases.[2-5] Chronic hepatitis C frequently evolves to cirrhosis, and a significant proportion of patients with liver cirrhosis will develop hepatocellular carcinoma (HCC).[6] Treatment of chronic hepatitis C with interferon alfa is effective in less than 50% of patients, and a high proportion of those who respond to the treatment relapse soon after interferon withdrawal.[7] It is clear that the elaboration of a vaccine against HCV and the development of new therapeutic methods are important goals for the future.

Cytotoxic T lymphocytes (CTL) have been shown to play a major role in the control of many viral diseases.[8-15] HCV infection has a strong tendency to chronicity suggesting that the CTL reaction against HCV antigens is poor or ineffective. Thus, the characterization of CTL epitopes from HCV proteins as well as the development of efficient ways of inducing CTL *in vivo* are important steps toward prevention and/or treatment of HCV infection.

To induce *in vivo* CTL against viral antigens, the use of recombinant viral vectors constitutes an attractive strategy. These vectors express recombinant proteins inside the cell, allowing endogenously synthesized antigens to be processed in a nonendosomal compartment and the derived peptides of 8-10 residues to be transported to the lumen of endoplasmic reticulum where they bind to class-I major histocompatibility complex molecules for presentation to CD8$^+$ CTL at the cell membrane. In fact, previous studies have shown that the expression of HCV antigens, using recombinant vaccinia virus, is an efficient means to induce specific CTL in mice.[16-18] While replication-competent vaccinia recombinants entail substantial risks in men,[19] replication-deficient adenoviruses do not appear to be hazardous for humans.[20] These recombinant viruses are able to express foreign antigens very efficiently inside nonpermissive cells without spreading the infection.[21,22] Based on these principles, we constructed a recombinant adenovirus containing core and E1 genes of HCV; we then studied its ability to express these proteins in mice and human cells and to induce a cytotoxic immune response in mice. As discussed in later paragraphs, our adenovirus was very effective both in expressing HCV transgenes and in stimulating specific CTL in mice against HCV antigens. Fine mapping of CTL epitopes using overlapping peptides allowed us to identify 6 peptides from core and one from E1 containing cytotoxic T cell determinants.

MATERIALS AND METHODS

Mice. Female BALB/c mice (range, 4-6 weeks old) were purchased from IFFA Credo (Barcelona, Spain). They were hosted in appropriated animal care facilities during the experimental period and were handled following the international guidelines required for experimentation with animals.

Cells and Viruses. Two hundred ninety-three cells[23] (European collection of animal cell cultures No: 85120602), derived from human embryonic kidney and stably transfected with the E1 region of the adenovirus type 5 (Ad5), were cultured in Glasgow's minimal essential medium with 8% fetal calf serum at 37°C and 5% CO_2. These cells were used for the generation and propagation of the recombinant adenoviruses.

P815 cells (ATCC TIB64) derive from a DBA/2 mouse mastocytoma and express *H-2*^{d} class-I molecules. EL-4 cells (ATCC TIB39) derive from a C57BL/6N mouse lymphoma and express *H-2*^{b} class-I molecules. Both EL-4 and P815 only express class-I but not class-II major histocompatibility molecules. These two cell lines were cultured in

Abbreviations: RAd, recombinant adenovirus; HCV, hepatitis C virus; CTL, cytotoxic T lymphocyte.

From the [1]Department of Medicine and Liver Unit, University Clinic and Medical School, University of Navarra, Pamplona, Spain; [2]the Department of Medicine, University of Wales College of Medicine, Cardiff, UK; and [3]the Department of Molecular Sciences, Wellcome Foundation LTD, Beckenham, Kent, UK.

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Address reprint requests to: J. Prieto, M.D., Departamento de Medicina Interna, Clínica Universitaria, Facultad de Medicina, Universidad de Navarra, C/Pio XII s.n., 31080 Pamplona, Spain.

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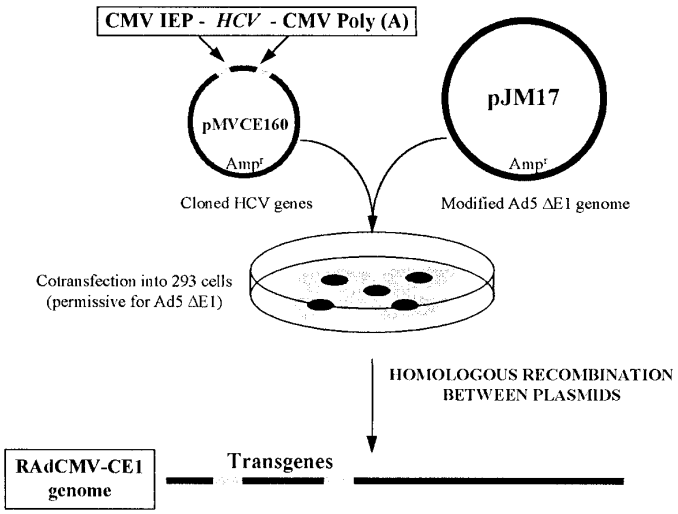

FIG. 1. Construction of RAdCMV-CE1. Plasmid pMVCE160, containing core and E1 sequences of HCV inside the CMV expression cassette and flanked by adenoviral sequences, was transferred together with pJM17 into 293 cells (an Ad5 ΔE1 permissive cell line). Recombination between homologous sequences from both plasmids permitted the recovery of a new virus (RAdCMV-CE1) with both HCV transgenes inserted into the genome.

RPMI-1640 medium plus 10% fetal calf serum, antibiotics, and 5×10^{-5} mol/L 2-mercaptoethanol at 37°C and 5% CO_2 and were used as target cells for cytotoxicity assays.

18Neo cells (kindly provided by Dr. Jay A. Berzofsky, Food and Drug Administration, Bethesda, MD) derive from BALB/c 3T3 fibroblasts and express *H-2*^{d} molecules. This cell line was cultured in Dulbecco's minimal essential medium with 10% fetal calf serum and antibiotics at 37°C and 5% CO_2.

Plasmids. Recombinant complementary DNA clones, generated from a chronic HCV carrier by polymerase chain reaction, were reconstructed into longer continuous sequences by ligation through natural restriction sites. One clone, pK155, contains sequences representing a genotype 1b entire core and E1 regions and terminates within the middle of the E2 gene. This clone was initially modified using synthetic oligonucleotides to provide a termination codon (TAA) and a novel *Xba*I site at the 3′terminus of the E1 gene. This allowed the entire core and E1 regions to be inserted as an *Xba*I fragment downstream of the cytomegalovirus major immediate early promoter (CMV IEP) into the transient expression vector pMV100.[22] The CMV IEP/HCV core and E1 expression cassette was then excised on a *Hin*d III fragment and inserted into the adenovirus recombination vector pMV60, essentially as described by Jacobs et al.[21] to generate pMVCE160. The integrity of pMVCE160 was confirmed by restriction endonuclease cleavage and nucleic acid sequence analysis of the insert.

Generation of Recombinant Adenoviruses. The recombinant adenovirus (RAd) RAdCMV-CE1 (RAdCMV-CE1) was generated using the technique of McGrory et al.[24] As shown in Fig. 1, plasmids pMVCE160 and pJM17 were cotransfected into 293 cells. pJM17 contains the complete Ad type 5 d1309 genome modified to exceed the adenovirus packaging limit. Homologous recombination between the two plasmids results in the transgene being inserted into the Ad genome in place of its E1 gene region. This decrease in the size of the Ad5 genome enables the generation of RAdCMV-CE1 encoding HCV core and E1.

RAdCMV-CE1 was propagated on 293 cells and purified in a CsCl isopicnic banding step. The viral band was dialyzed against 0.01 mol/L Tris, pH 8. At this stage, the virus was kept in aliquots at −80°C.

All transfections were carried out using the calcium phosphate precipitation method as described by F. Graham et al.[25]

The construction of recombinant adenovirus RAdCMV-LacZ containing the LacZ reporter gene has already been described elsewhere.[22] Transduction of both mouse and human fibroblasts using this construct was very efficient since at a relatively low multiplicity of infection (10) the expression of the reporter gene was observed in over 70% of the cells.

Immunofluorescence. Immunofluorescence experiments in both human and murine fibroblasts infected with RAdCMV-CE1, RAdCMV-LacZ, or non-infected were carried out using standard techniques. Briefly, fibroblasts were fixed with methanol, preincubated with 3% bovine serum albumin in phosphate buffer saline to block nonspecific binding and were then incubated with the primary antibody, either human anti-HCV positive sera, a monoclonal anti-core antibody (provided by Dr. E. Martinez, University of Navarra, Pamplona, Spain), or a monoclonal anti-E1 antibody (anti-E1 mabIGH-201, provided by Dr. G. Maertens, Innogenetics, Ghent, Belgium) at different dilutions (range, 1:20-1:100). Anti-human or anti-mouse secondary antibodies fluorescein isothiocyanate conjugated were added at 1:400 and 1:1,000 dilutions, respectively. After incubation, preparations were mounted and observed in an ultraviolet microscope. Extensive cell washes were performed between each incubation period.

Peptide Synthesis. Peptides were synthesized by the solid phase method of Merrifield,[26] using the Fmoc alternative.[27] Twenty-four 14-mer and thirty-six 15-mer (10 amino-acid overlap) peptides spanning the whole sequence of core and E1 proteins of HCV genotype 1b were synthesized using a multiple solid phase peptide synthesizer[28] and were used without further purification. Seven 10-mer (9 amino-acid overlap) peptides were also synthesized using the same method to map the minimal epitope inside the 15-mer peptide 312-326.

CTL Generation by Immunization With RAdCMV-CE1. Six-week old female mice were immunized intraperitoneally with 10^8 pfu of RAdCMV-CE1 virus in 500 μL of phosphate-buffered saline. Ten days after immunization, spleens were removed and homogenized, cells were cultured *in vitro* in 24-well plates at 3×10^6 cells/mL in the presence of different peptide pools (Tables 1 and 2). A final concentration of 5 μg/mL of each peptide, dissolved in RPMI-1640 medium supplemented with 10% fetal calf serum, antibiotics, and 5×10^{-5} mol/L of 2-mercaptoethanol was used. Six days later, cytotoxic activity was measured.

^{51}Cr Release Cytotoxic Assay. CTL activity was measured using a conventional cytotoxicity assay.[29] Assays were done in triplicate at different effectors to target cell ratios (as indicated in each experiment); the spontaneous release was in all cases below 25% of total release. A positive response was considered when the percentage of specific lysis against target cells incubated with one specific peptide exceeded the percentage of lysis obtained by calculating means +

TABLE 1. Peptide Mixtures Corresponding to the Core Region of HCV Used for *In Vitro* Restimulation of BALB/c Mice Spleen Cells Immunized With RAdCMV-CE1

Aminoacid Position	Aminoacid Sequence	
1-14	MSTNPKPQRKTKRN	
39-52	RRGPRLGVRATRKT	
53-66	SERSQPRGRRQPIP	Pool 1
71-84	PEGRAWAQPGYPWP	
85-98	LYGNEGMGWAGWLL	
99-112	SPRGSRPSWGPTDP	
113-126	RRRSRNLGKVIDTL	Pool 2
169-182	LPGCSFSIFLLALL	
178-191	LLALLSCLTIPASA	
6-19	KPQRKTKRNTNLRP	
41-54	GPRLGVRATRKTSE	
57-70	QPRGRRQPIPKARQ	Pool 3
76-89	WAQPGYPWPLYGNE	
90-103	GMGWAGWLLSPRGS	
132-145	DLMGYIPLVGAPLG	
151-164	LAHGVRVLEDGVNY	Pool 4
173-186	SFSIFLLALLSCLT	
11-24	TKRNTNLRPQDVKF	
34-47	VYLLPRRGPRLGVR	
46-59	VRATRKTSERSQPR	Pool 5
109-122	PTDPRRRSRNLGKV	
37-50	LPRRGPRLGVRATR	
48-61	ATRKTSERSQPRGR	Pool 6
165-178	ATGNLPGCSFSIFL	

TABLE 2. Peptide Mixtures Corresponding to the E1 Region of HCV Used for *In Vitro* Restimulation of BALB/c Mice Spleen Cells Immunized With RAdCMV-CE1

Aminoacid Position	Aminoacid Sequence*	
192-206	YEVRNVSGIYHVTNDA	
207-221	CSNSSIVYETADMINA	
222-236	HTPGCVPCVREGNSSA	Pool 1
237-251	RCWVALTPTLAAKDAA	
252-266	SIPTATIRRHVDLLVA	
267-281	GAAAFCSAMYVGDLCA	
282-296	GSVFLVSQLFTFSPRA	
297-311	RHQTVQDCNCSIYPGA	
312-326	HVSGHRMAWDMMMNWA	Pool 2
327-341	SPTAALVVSQLLRIPA	
342-356	QAVVDMVAGAHWGVLA	
357-371	AGLAYYSMVGNWAKVA	
197-211	VSGIYHVTNDCSNSSA	
212-226	IVYETADMIMHTPGCA	
227-241	VPCVREGNSSRCWVAA	Pool 3
242-256	LTPTLAAKDASIPTAA	
257-271	TIRRHVDLLVGAAAFA	
272-286	CSAMYVGDLCGSVFLA	
287-301	VSQLFTFSPRRHQTVA	
302-316	QDCNCSIYPGHVSGHA	
317-331	RMAWDMMMNWSPTAAA	Pool 4
332-346	LVVSQLLRIPQAVVDA	
347-361	MVAGAHWGVLAGLAYA	
362-376	YSMVGNWAKVLVVMLA	
202-216	HVTNDCSNSSIVYETA	
217-231	ADMIMHTPGCVPCVRA	
232-246	EGNSSRCWVALTPTLA	Pool 5
247-261	AAKDASIPTATIRRHA	
262-276	VDLLVGAAAFCSAMYA	
277-291	VGDLCGSVFLVSQLFA	
292-306	TFSPRRHQTVQDCNCA	
307-321	SIYPGHVSGHRMAWDA	
322-336	MMMNWSPTAALVVSQA	Pool 6
337-351	LLRIPQAVVDMVAGAA	
352-366	HWGVLAGLAYYSMVGA	
367-381	NWAKVLVVMLLFAGVA	

* The underlined A was added for synthesis convenience.

2.6 SD ($P < .01$) of the values from control wells containing unpulsed target cells.

When peptides were used, target cells P815 (*H-2*^{d} restricted) or EL-4 (*H-2*^{b} restricted) were radiolabelled with 50 μCi of $Na_2{}^{51}CrO_4$ per 10^6 cells for one hour at 37°C, washed twice, and incubated with 5 μg/mL of the relevant peptide.

When recombinant adenoviruses were used to express proteins inside target cells, these, 18Neo (*H-2*^{d} restricted), were infected with either RAdCMV-CE1 or RAdCMV-LacZ for 36 hours before the assays, to allow gene expression. Subsequent radiolabeling of the cells was carried out, as previously described.

Abrogation of the CTL Response by Anti-CD4 and Anti-CD8 Monoclonal Antibodies. L3T4 rat anti-mouse hybridomas GK1-5 (CD4 specific) and H35.17.2 (CD8 specific) (kindly provided by Dr. C. Leclerc, Institute Pasteur, Paris, France) were used to obtain anti-CD4 and anti-CD8 monoclonal antibodies. Ascitic fluid was obtained from nude mice, was pristane primed, and was injected intraperitoneally with 10^6 hybridoma cells. Antibodies were prepared by precipitation with ammonium sulphate followed by dialysis against phosphate-buffered saline. Protein concentration was assessed by optical density measurement at 280 nm. In the CTL response-blocking experiments, the chromium release assay was carried out in the presence of anti-CD4 or anti-CD8 antibodies (10 μg/mL of final concentration) plus complement from rabbit serum (final dilution, 1:20).

RESULTS

Expression of HCV Proteins in Human Fibroblasts Using RAdCMV-CE1. To know whether the replication-deficient recombinant adenovirus containing core and E1 proteins from HCV (RAdCMV-CE1) were able to express the recombinant proteins, we performed Western transfer analysis of cell extracts from mice (18Neo) and human fibroblasts (from skin biopsies) infected with RAdCMV-CE1 using seropositive human sera as source of primary antibodies. A band sized at 22 kd corresponding to the core protein and several bands sized from 30 to 35 kd corresponding to E1 protein isoforms[30,31] were detected (data not shown).

To confirm that RAdCMV-CE1 express HCV core and E1 proteins in the infected cells, immunofluorescence experiments using anti-HCV–positive human sera, a monoclonal anti-core antibody, and a monoclonal anti-E1 antibody were also performed. Figure 2 shows the indirect immunofluorescent staining of mouse and human fibroblasts, infected with RAdCMV-CE1, with RAdCMV-LacZ, or noninfected, incubated with human seropositive serum, monoclonal anti-core antibody, or monoclonal anti-E1 antibody. As observed in the figure, a dotted coarse granular pattern of specific staining was noted when the monoclonal anti-core antibody was used as the primary antibody (Fig. 2D), while a diffuse punctuated cytoplasmic pattern was found when using anti-E1 antibody (Fig. 2E). A combination of both patterns was found when human anti-HCV positive serum was employed, suggesting the recognition of both core and E1 proteins by this serum

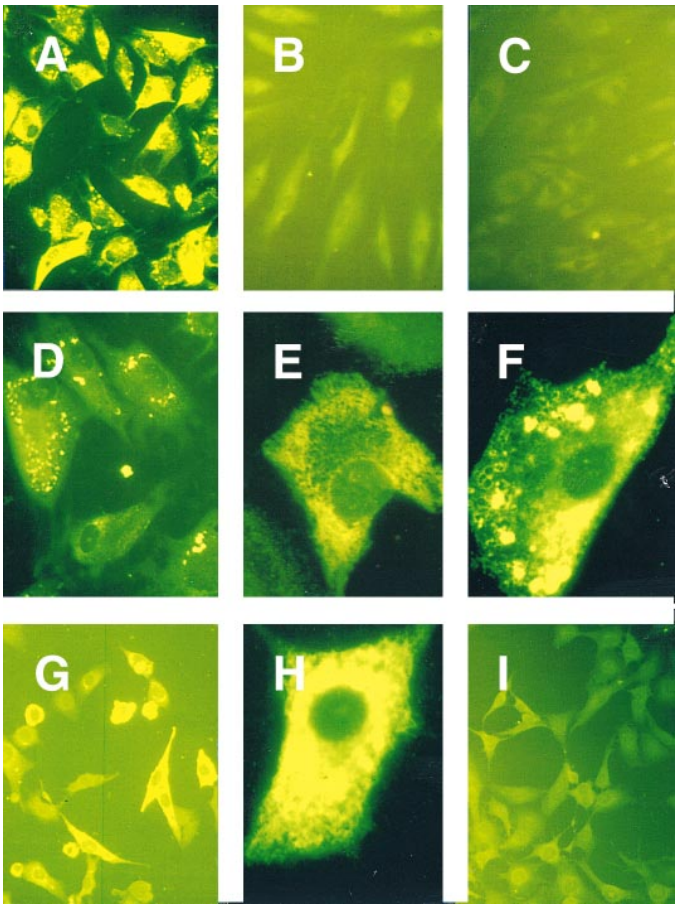

FIG. 2. Indirect immunofluorescence of HCV proteins expressed by recombinant defective adenovirus in human or mouse fibroblasts. Human fibroblasts (A-F) infected with RAdCMV-CE1 (A, D, E, and F), RAdCMV-LacZ (B), and noninfected (C) were incubated with human anti-HCV positive serum (A, B, C, and F), with a mouse monoclonal anti-core antibody (D) or with a mouse monoclonal anti-E1 antibody (E). Mouse fibroblasts (G, H, and I) infected with RAdCMV-CE1 (G and H) or RAdCMV-LacZ (I) were incubated with human anti-HCV positive serum. Magnification was ×200 in A, B, C, G, and I; ×400 in D; and ×1000 in E, F, and H.

(Fig. 2A, 2F, 2G, and 2H). These experiments indicate a high efficiency of expression of recombinant HCV proteins by our adenoviral vector. The fine punctuate staining of the infected cells using the anti-E1 antibody and the granular pattern observed with anti-core suggest the association of these recombinant proteins with cell membranes, as previously described.[30] These two distinct immunofluorescence patterns have also been observed by others.[32,33]

Induction of CTL in Mice Using RAdCMV-CE1. RAdCMV-CE1 was used to immunize BALB/c mice in an attempt to induce a CTL response against HCV structural proteins. The mice were immunized by intraperitoneal route with 10^8 pfu of virus; after 10 days their spleens were removed. In order to assess CTL activity against core and E1, these spleen cells were incubated in the presence of pools of overlapping synthetic peptides (Tables 1 and 2) spanning the whole sequence of these proteins. After six days in culture, CTL activity was tested against P815 cells incubated with each specific peptide, using a chromium release assay. Figures 3A and 3B show that the immunization of mice with the recombinant resulted in a CTL response that was directed against P815 target cells incubated with six core peptides [34-47, 37-50, 39-52, 132-145, 165-178, and 173-186] and a single E1 peptide [312-326]. The first three core peptides most probably correspond to two determinants: one spanning residues 35-44 (contained in peptide 34-47) already described by Cerny et al. in chronic HCV infected patients,[34] and the other one spanning residues 41-49 (contained in peptides 37-50 and 39-52) as described by Koziel et al.[35] in patients with chronic hepatitis C. Core peptides 132-145 and 173-186 have also been found to function as CTL determinants in humans.[18,34,36] These results indicate the importance of the highly conserved core protein as a target for cytotoxic T cells and they also illustrate the importance of mice studies to predict cytotoxic epitopes in humans.[37] While most CTL determinants that were found in the core protein were previously described, the E1 epitope encountered in this study was novel.

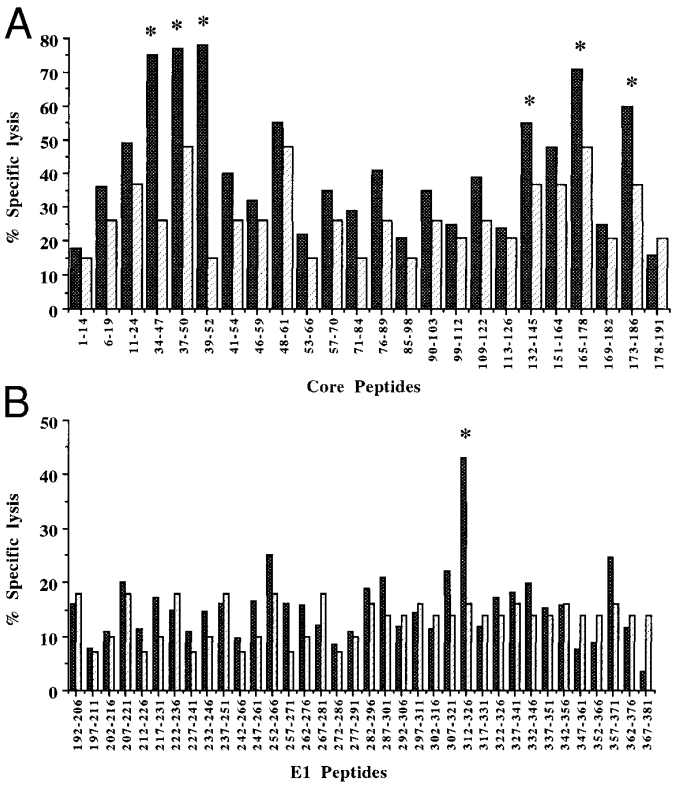

FIG. 3. CTL activity (^{51}Cr release assay) of spleen cells from BALB/c mice immunized with recombinant adenovirus RAdCMV-CE1 against P815 target cells preincubated with overlapping synthetic peptides spanning HCV core (A) and E1 (B) proteins. Before the assay splenocytes were restimulated *in vitro* with pools of peptides (Tables 1 and 2) as indicated in Materials and Methods. For each peptide, a double cytotoxicity value is shown: the specific lysis obtained when the peptide is added to target cells (■); and the background lysis of unpulsed target cells (□). A positive response was considered when the percentage of specific lysis of target cells incubated with the peptide exceeded means ± 2.6 SD ($P < .01$) of the values from the control wells containing unpulsed target cells. Effector to target cell ratios used were: 50:1 for core derived peptides and 16.6:1 for E1 peptides. *Positive cytotoxic responses.

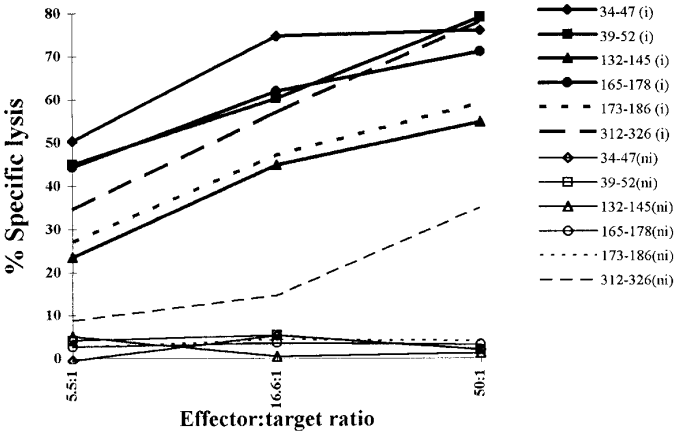

FIG. 4. Experiment to rule out that the observed cytotoxic T cell response was not induced *in vitro.* Immunized [i] and non-immunized [ni] BALB/c mice spleen cells were restimulated *in vitro* for six days with peptides from core (34-47, 39-52, 132-145, 165-178, and 173-186) and E1 (312-326). They were then tested against P815 cells sensitized with individual core and E1 peptides. Results show that only mice previously immunized with RAdCMV-CE1 display cytotoxic activity against the target cells.

As observed in Fig. 3A, background cytolytic activity was relatively high for those core peptides corresponding to pools 4 (peptides 11-24, 132-145, 151-164, and 173-186) and 6 (peptides 37-50, 48-61, and 165-178). However the difference between specific lysis and background lysis defining positive responses (>2.6 SD of the mean of cytotoxicity values in wells containing unpulsed target cells) was maintained in confirmatory experiments for the different core peptides tested (data not shown). This high background might be related to the presence in the pool of peptides possessing T-helper cell determinants able to activate interleukin-2 production thus generating nonspecific CTL activity during *in vitro* restimulation.[38]

We analyzed whether the observed cytotoxic T-cell responses against different epitopes from core and E1 were indeed induced *in vivo* by the recombinant adenovirus and not by the *in vitro* incubation of spleen cells with the corresponding peptide. To this we compared the cytotoxic activity of lymphocytes from immunized and nonimmunized mice, stimulated *in vitro* with the peptides eliciting positive cytotoxic responses, against target cells sensitized with these same peptides. Figure 4 shows that the cytotoxic activity of cells from mice immunized with RAdCMV-CE1 was significantly higher than that observed when using cells from nonimmunized mice, indicating the ability of RAdCMV-CE1 to induce cytotoxic T cells in animals infected with this vector.

Characterization of CTL Response. As mentioned, most of the epitopes we found in core had been previously described and their minimal CTL epitopes identified,[18,34,35,36] while the E1 312-326 peptide was novel. We selected this peptide, which corresponds to a highly conserved motif among all HCV isolates, to further characterize the CTL response induced by our adenoviral recombinant.

Several studies (e.g., references 39 and 40) have indicated that the usual length of processed antigen, found complexed

TABLE 3. Recognition of 10-mer Overlapping Peptides From 312-326 by the CTL Induced in BALB/c Mice With RAdCMV-CE1

Peptide*		% Lysis Ratio 50:1	% Lysis Ratio 16:1
HVSGHRMAWDMMMNWA	(312-326)	70	41
HVSGHRMAWA		30	18
VSGHRMAWDA		57	35
SGHRMAWDMA		50	35
GHRMAWDMMA		45	37
HRMAWDMMMA		31	24
RMAWDMMMNA		25	22
MAWDMMMNWA		26	17
None		22	13

* The underlined A was added for synthesis convenience.

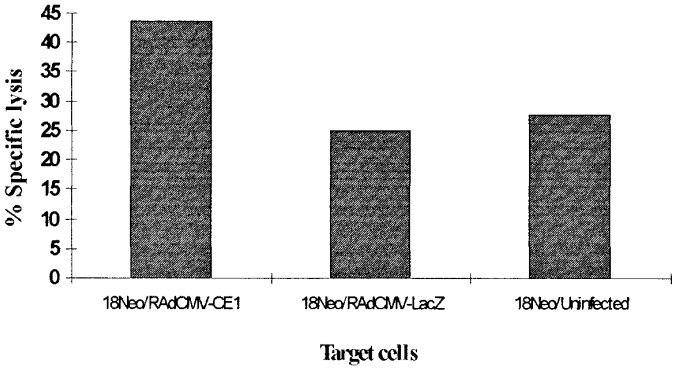

FIG. 5. Recognition of endogenously synthesized antigens. 18 Neo cells previously infected with RAdCMV-CE1, the control adenovirus RAdCMV-LacZ or non-infected were used as targets in a chromium release assay, using immunized BALB/c mice spleen cells which had been restimulated with peptide 312-326. Effector to target cell ratio for this experiment was 25:1.

to class-I molecules on antigen presenting cells, is between 8 and 10 amino acids. Thus, to define the minimal CTL epitope present in peptide 312-326, we synthesized the overlapping 10-mer peptides covering its entire sequence (Table 3). These peptides were used to preincubate P815 target cells which were then assessed for their susceptibility to lysis by CTL derived from immunized mice. Table 3 shows that the determinant is present in decapeptides 2, 3, and 4, indicating that the minimal epitope comprises the amino acid sequence GHRMAWDM.

The minimal determinant identified in Table 3 corresponds to a highly conserved region of E1 among different genotypes.[41] However, the region flanking the N-terminus of this determinant does vary between genotypes. To study the effect of these amino acid changes on the CTL activity induced with RAdCMV-CE1 expressing the sequence of genotype 1b, we synthesized peptide 312-326 corresponding to HCV genotype 1a and compared the recognition of P815 target cells preincubated with peptide sequences 312-326 from genotypes 1b and 1a. Table 4 shows that the amino acid mutations at positions 313 and 314 (which lay outside the minimal epitope) do not prevent the recognition of target cells by CTL.

We also investigated whether cytotoxic T cells from immunized mice, restimulated *in vitro* with peptide 312-326, were able to recognize not only target cells incubated with this peptide but also the determinant derived from the naturally processed viral protein presented on the membrane of cells infected with RAdCMV-CE1. As controls we used 18Neo cells infected with a recombinant adenovirus expressing β-galactosidase (RAdCMV-LacZ) and noninfected cells. Figure 5 shows that 18Neo cells infected with RAdCMV-CE1, therefore exhibiting native HCV E1 processed peptides complexed to major histocompatibility complex class-I molecules, were predominantly recognized and lysed whereas the same cells infected with the control virus or that were non-infected were not.

To further characterize the nature of the CTL response to RAdCMV-CE1, the phenotype of the induced lymphocytes was analyzed by carrying out the cytotoxicity assay in the presence of either anti-CD4 or anti-CD8 antibodies plus complement. CTL activity against P815 target cells incubated with peptide 312-326 was abrogated by anti-CD8 antibodies plus complement but not by the addition of anti-CD4 plus complement or by complement alone (Fig. 6A). This indicates that the CTL induced by immunization with RAdCMV-CE1 corresponds to cells with the phenotype $CD4^-$ $CD8^+$.

Experiments were also performed to map the major histocompatibility complex-restriction of the CTL induced by immunization with RAdCMV-CE1. To this aim we compared CTL-mediated lysis using class-I matched target cells from an *H-2*^{d} background (P815) and class-I mismatched cells from an *H-2*^{b} background (EL-4). Both cell types were preincubated with peptide 312-326 and then analyzed in a cytotoxicity assay against lymphocytes from BALB/c mice (*H-2*^{d}) immunized with RAdCMV-CE1. Figure 6B shows that only class-I matched target cells were lysed (P815) indicating that the CTL response to RAdCMV-CE1 was *H-2*^{d} restricted.

To study the kinetics of the specific cytotoxic T-cell activity generated by immunization with RAdCMV-CE1, further experiments were performed in which mice were killed between 10 and 100 days, postintraperitoneal injection of the recombinant. We found that spleen cells isolated from mice at 10, 40, and 100 days postimmunization displayed similar cytotoxicity when incubated with target cells sensitized with peptide 312-326 (data not shown). Because RAdCMV-CE1 is a nonreplicating adenoviral vector, these findings indicate that the primary CTL response induced upon a single immunization can be sustained for at least 100 days.

TABLE 4. Cross Recognition of Peptides From Genotype 1a and 1b by the CTL Induced With RAdCMV-CE1

Peptide*	Genotype	% Lysis Ratio 15:1	% Lysis Ratio 5:1
HVSGHRMAWDMMMNWA	1a	60	29
-IT----------A	1b	48	26
None		27	11

* The underlined A was added for synthesis convenience.

DISCUSSION

Following infection with a virus, endogenously synthesized viral proteins are processed to yield short peptides which bind to major histocompatibility complex class I molecules for presentation on the cell membrane to $CD8^+$ CTLs which,[15] together with $CD4^+$ helper T cells, are believed to play a critical role in the clearance of viral infections.[11,14,42,43] Direct delivery of fragments of DNA encoding viral proteins to the cells can reproduce this same chain of events, leading to the induction of a protective antiviral immunity.[8,10,21,44-46]

Recombinant viruses constitute an efficient means for DNA transfer to cells both *in vivo* and *in vitro*. When transgenes are placed in these vectors under the drive of a strong promoter such as cytomegalovirus immediate early promoter, as in this study, it is possible to achieve a high level of expression of the recombinant proteins. Several reports[8,10,44-46] have shown that infection of animals with recombinant viruses encoding transgenic viral proteins is able to induce protective immunity to the virus of interest in a number of systems.

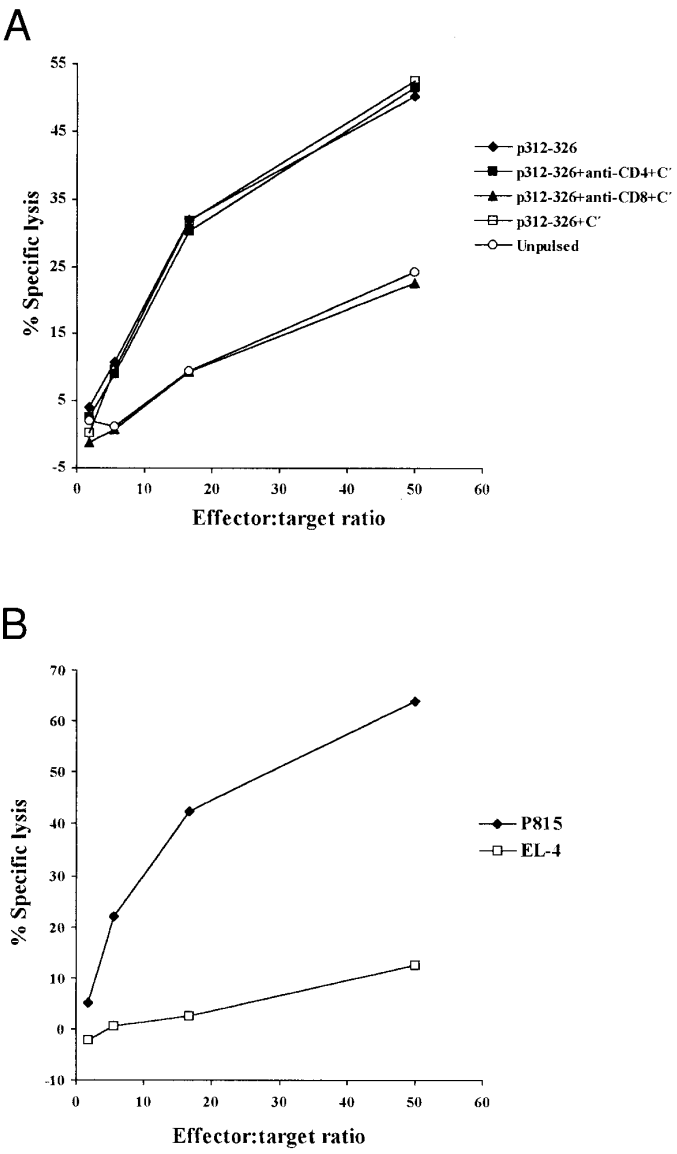

FIG. 6. Characterization of CTL induced by RAdCMV-CE1 against epitope 312-326 from E1. (A) Surface phenotype. The chromium release assay was carried out using P815 cells pulsed or unpulsed with peptide 312-326. The assay was carried out under the conditions shown, in the presence of anti-CD4 or anti-CD8 plus complement or complement alone. (—◆—), p312-326; (—■—), p312-326 + anti-CD4 + C^1; (—▲—), p312-326 + anti-CD8 + C^1; (—□—), p312-326 + C^1; and (—○—), unpulsed. (B) Major histocompatibility class I restriction of induced CTL was assessed using P815 (*H-2*^{d}) or EL-4 (*H-2*^{b}) target cells pulsed with peptide 312-326. This figure shows that only class-I matched target cells (P815) were lysed, indicating that the CTL response to RAdCMV-CE1 was *H-2*^{d} restricted. (—◆—), P185; and (—□—), EL-4.

It is not surprising, therefore, that this strategy has been considered in the development of vaccines against a diversity of viral agents.[45,47,48] Because of the safety for humans of disabled recombinant viral vectors,[20] we used a replication-deficient adenoviral construct in order to express HCV proteins *in vivo* and to induce a cytotoxic T cell response against these antigens. Our immunofluorescence studies show the ability of adenoviral vectors to express recombinant HCV proteins at high levels and in a manner that is readily recognized by antibodies elicited during natural infection.

Shirai et al.[17] have reported that the administration of a recombinant vaccinia virus expressing HCV core protein to mice resulted in the generation of a CTL response against a conserved epitope of this protein. In the present work, we show that the replication-deficient recombinant adenovirus RAdCMV-CE1 was able to express HCV core and E1 proteins both in mice and human cells, and that this vector induced in mice a strong CTL response against different epitopes from core (peptides 34-47, 37-50/39-52, 132-145, 165-178, and 173-186) and E1(312-326).

Under the conditions of our immunization protocol (one single injection of the vector) we were able to induce cellular but not humoral immunity to HCV proteins. However it should be mentioned that immunization with repeated injections of a recombinant defective adenovirus expressing HCV structural proteins under the control of a Roux sarcoma a promoter has succeeded in inducing humoral immunity to HCV.[49]

One of the dominant cytotoxic T-cell epitopes that we have found in core was encompassed by two overlapping peptides: 37-50 and 39-52. This cytotoxic T-cell determinant may correspond to epitope 41-49, described by Koziel et al.,[35] as recognized by liver infiltrating lymphocytes from patients with chronic HCV infection. Peptide 34-47 contains, based on predictions,[50,42] a different epitope which has also been signaled by Cerny et al.[34] and by Battegay et al.[36] as recognized by peripheral blood lymphocytes in HLA-A2 positive chronic hepatitis C patients. Of interest, two out of the other three core determinants (peptides 132-145 and 173-186) had also been described by Cerny et al.[34] (both peptides), by Battegay et al.,[36] (peptide 173-186) and by Shirai et al.[17,18] (peptide 132-145) as targets of CTL responses by peripheral blood lymphocytes from HLA-A2 positive patients with chronic hepatitis C. In addition to these observations, results from our laboratory (Lasarte et al., unpublished observations, January 1996) using techniques based on competition for binding to HLA-A2, have shown that peptide 165-178 also contains an HLA-A2 binding motif. On the other hand, Shirai et al.[17] have identified core peptide 133-142 as a cytotoxic T-cell determinant in *H-2*^{d} mice. Minimal epitopes of some of the above described CTL determinants have been characterized.[17,35] All of these results emphasize the importance of core as a target of cytotoxic attack.

Although there are data indicating that cellular immunity to core appears to play a role in the control of HCV infection,[51] there is also evidence suggesting that immunity to envelope proteins may contribute to viral clearance.[52] Also we have observed that patients with chronic hepatitis C who cleared the virus after interferon therapy showed proliferative T cell responses to peptides from E1, whereas there was little T-cell recognition of these peptides in those which remained viremic after treatment.[53] In the present study, we found that while the cytotoxic activity induced by immunization with RAdCMV-CE1 was directed against a diversity of epitopes in core, a single CTL epitope was found in E1. While most of the cytotoxic T-cell determinants found in core had been previously described, the determinant encountered in E1 (peptide 312-326) was novel. This peptide was of interest since in a previous study in patients with chronic hepatitis C, we found that it also contains a helper T-cell determinant.[53] In fact the immunization of mice with this peptide induced a specific CTL response of similar intensity as that elicited with RAdCM1V-CE1 (Lasarte et al., unpublished observations, March 1995). Moreover, the minimal CTL epitope of this determinant (peptide GHRMAWDM) corresponds to a highly conserved region among all HCV isolates, which increases its value as one possible candidate for immunogen in vaccine designs.

Further characterization of the response to this determinant disclosed that the cytotoxic activity was mediated by $CD8^+$ T lymphocytes, was *H-2*^{d} restricted, and was long lasting. In this respect it is worth noting that one of the advantages of using recombinant defective adenoviruses is that they permit a relatively long duration of the expression of the recombinant proteins after a single injection of the vector.[44] It

is well known that despite low levels of expression of adenoviral proteins, recombinant defective adenoviruses induce a cytotoxic response directed against the vector, which tends to eliminate the transduced cells and limits the expression of the transgenes to a few weeks.[54,55] This time, however, the timing is long enough to facilitate the development of an enduring immunity against the viral proteins encoded by the HCV genes inserted into the adenoviral vector. In this study, the intensity of the cytotoxic T-cell response was maintained at the same level for at least 100 days after the injection of the vector.

HCV infection is characterized by a strong chronic tendency. Although, as described previously, CTL against viral antigens have been found in chronic HCV carriers, the cytotoxic T-cell response in this infection appears to be weak or inefficient.[56] In fact the frequency of CTL precursors against determinants present in core, envelope proteins, or nonstructural proteins found in patients with chronic hepatitis C is between $1/10^6$ and $1/10^5$.[34] The frequency of circulating CTL precursors against HCV antigens in the general population is between 10 to 100 times lower. These figures should be compared with the frequency of CTL precursors against human or cytomegalovirus found in asymptomatic human immunodeficiency virus or cytomegalovirus-infected patients which is 1 to 2×10^3 and 1 to 5×10^3, respectively.[57,58] It seems possible, therefore, that procedures aimed at increasing the frequency of CTL precursors in non-infected individuals might provide protection against HCV infection. Recombinant defective adenoviruses expressing HCV structural proteins, such as the one used in the present study, appear to be good tools to achieve this goal. These vectors should be considered as a possible vaccine if studies in chimpanzees demonstrate protective effects.

HCV is very efficient in developing strategies to escape the immune system and to produce chronic infection. One of the mechanisms is the mutation of relevant cytotoxic T cell determinants,[56,59] and another is, as mentioned, inducing a CTL response of low intensity which, although capable of producing cell damage, is inefficient to clear the virus. The low intensity of the CTL response against HCV antigens is possibly related to the low level of expression of viral proteins and the low level of viremia which characterizes this infection. Recombinant viral vectors permit expression at a high level of transgenic viral proteins and stimulate cytotoxic T-cell responses against multiple epitopes at the same time. Under these conditions it might be difficult for the virus to escape the cytotoxic attack. Thus, there are grounds to think that vectors such as RAdCMV-CE1 might be a useful complementary therapy in the treatment of chronic hepatitis C. This disease responds poorly to interferon, and a high proportion of those who respond relapse after interferon withdrawal.[7] The stimulation of cytotoxic T-cell activity by means of recombinant defective adenoviruses, after reducing the viral load with interferon, might possibly have the beneficial effect of reducing the risk of posttherapy relapse.

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