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-2d restricted, lasting for at least 10 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. Infection with HCV leads to chronic hepatitis in about 80% of cases. Chronic hepatitis C frequently evolves to cirrhosis, and a significant proportion of patients with liver cirrhosis will develop hepatocellular carcinoma (HCC). 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. 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. HCV infection has a strong tendency to chronicity suggesting that the CD8+ 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. While replication-competent vaccinia recombinants entail substantial risks in men, replication-deficient adenoviruses do not appear to be hazardous for humans. These recombinant viruses are able to express foreign antigens very efficiently inside nonpermissive cells without spreading the infection. 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 housed 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 (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 cultivated in Glasgow’s minimal essential medium with 8% fetal calf serum at 37°C and 5% CO2. 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-2d class-I molecules. EL-4 cells (ATCC TIB39) derive from a C57BL/6N mouse lymphoma and express H-2d 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 lymphocytes.

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A cytotoxic T lymphocyte (CTL) assay was used to assess the cytotoxic potential of the immune response to the transgene in vivo. CTL lines were generated by immunization with RAdCMV-CE1 as described previously. Mice were immunized with RAdCMV-CE1 and spleen cells were harvested 14 days later. The splenocytes were restimulated with the transgene-expressing cells in vitro and the specific CTL activity was measured using a conventional 6-h cytotoxicity assay. The presence of specific cytotoxic activity was detected by a positive response in the 6-h assay. The percentage of specific lysis against target cells incubated with one specific peptide exceeded the percentage of lysis obtained by calculating means +

**Immunofluorescence.** Immunofluorescence experiments in both human and murine fibroblasts (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 antimouse 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, using Fmoc alternative. 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 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 10⁵ 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* at 24-well plates at 3 × 10⁶ 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 × 10⁻³ mol/L of 2-mercaptoethanol was used. Six days later, cytotoxic activity was measured.

**Cr Release Cytotoxic Assay.** CTL activity was measured using a conventional cytotoxicity assay. Assays were done in triplicate at different effectors to target cell ratios (as indicated in each experiment) as 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 | MEITNPKKQRTKKN |
| 39-52 | RGRGPLGVRATKTE |
| 53-66 | SERSAPRQRQIP |
| 71-84 | PECGWQAGCPYWP |
| 85-98 | LIGNEGMMWQL |
| 99-112 | SPSGRSPSWGPTD |
| 113-126 | RRSSRSNLKVDDT |
| 169-182 | LFCGCSFSIFLLLAL |
| 178-191 | LLALLSCLTFIPA |
| 6-19 | KRPQRTKNTNLKPR |
| 41-54 | GPRGLVRATKRS |
| 57-70 | QAPRQRQIPFARQ |
| 76-89 | WAPQPPWPVLGNE |
| 90-103 | GCMQAGMLSLPGS |
| 132-145 | DLMYTIPLVGAPLG |
| 151-164 | LAMGVRLLEDVNV |
| 173-186 | SFIRSIFLLALCCT |
| 11-24 | TKRTNLRKPDQWFK |
| 34-47 | VYLLPRFGRLGVR |
| 46-59 | VRATKRSQHESP |
| 59-70 | LPRFGRFGRVTR |
| 46-61 | ATATKRSQHESP |
| 90-103 | GCMQAGMLSLPGS |
| 132-145 | DLMYTIPLVGAPLG |
| 151-164 | LAMGVRLLEDVNV |
| 173-186 | SFIRSIFLLALCCT |
| 11-24 | TKRTNLRKPDQWFK |
| 34-47 | VYLLPRFGRLGVR |
| 46-59 | VRATKRSQHESP |
| 59-70 | LPRFGRFGRVTR |
| 46-61 | ATATKRSQHESP |
| 90-103 | GCMQAGMLSLPGS |
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 immunofluorescence 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.

### 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 were detected (data not shown).

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

<table>
<thead>
<tr>
<th>Aminoacid Position</th>
<th>Aminoacid Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>192-206</td>
<td>YEVMNVSGYHYTVDQA</td>
</tr>
<tr>
<td>207-221</td>
<td>CSNIS1YTVSIMNHN</td>
</tr>
<tr>
<td>222-236</td>
<td>HTPCVPVCRGNSSA</td>
</tr>
<tr>
<td>237-251</td>
<td>RCVWALVTPLAAXDQA</td>
</tr>
<tr>
<td>252-266</td>
<td>SIPTATIRKHVVOLLVQA</td>
</tr>
<tr>
<td>267-281</td>
<td>GAAFCSAMYVVHLGVA</td>
</tr>
<tr>
<td>282-296</td>
<td>GSVLVLQSGTFTSPR</td>
</tr>
<tr>
<td>297-311</td>
<td>HQTVQDCNSIYPQA</td>
</tr>
<tr>
<td>312-326</td>
<td>HVGGSRNMNNMNN</td>
</tr>
<tr>
<td>327-341</td>
<td>SPTAAYVQLGLLRIPA</td>
</tr>
<tr>
<td>342-356</td>
<td>QAQVDVMAHAGKVL</td>
</tr>
<tr>
<td>357-371</td>
<td>AGLAYSMYNVNAXVQA</td>
</tr>
<tr>
<td>197-211</td>
<td>VSGYVYTVCTDNSNSSA</td>
</tr>
<tr>
<td>212-226</td>
<td>IVYETADMMHPTGCQA</td>
</tr>
<tr>
<td>227-241</td>
<td>VPCVRENSGRCSWA</td>
</tr>
<tr>
<td>242-256</td>
<td>LTPAALAKDSIASPTAA</td>
</tr>
<tr>
<td>257-271</td>
<td>TIRHNYDVLGAAPFA</td>
</tr>
<tr>
<td>272-286</td>
<td>CSAMYVVGLCGQSVFDQA</td>
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<tr>
<td>287-301</td>
<td>VGQLFTSPFRMQTVQA</td>
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<tr>
<td>302-316</td>
<td>QCDCNIS1YPPGHSGHA</td>
</tr>
<tr>
<td>317-331</td>
<td>RMANDMNNMNNWSTPA</td>
</tr>
<tr>
<td>322-346</td>
<td>LVQLCQRIPQAVWQA</td>
</tr>
<tr>
<td>347-361</td>
<td>NYGASRWYGLAAY</td>
</tr>
<tr>
<td>362-376</td>
<td>YMVSMNNAXAKVWQLA</td>
</tr>
</tbody>
</table>

* 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 P315 (H-2d restricted) or EL-4 (H-2d restricted) were labelled with 50 μCi of Na214CrO4, per 106 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-2d restricted), were infected with either RAdCMV-CE1 or RAdCMV-LacZ for 36 hours before the assays, to allow gene expression. Subsequent radiolabelling 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 106 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).

**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 were detected (data not shown).

FIG. 2. Indirect immunofluorescence of HCV proteins expressed by recombinant 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.
Immunized with recombinant adenovirus RAdCMV-CE1 against P815 target. Characterization of CTL Response. AID Hepa 0008 / 5p1c

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 10^9 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, and the other one spanning residues 41-49 (contained in peptides 37-50 and 39-52) as described by Koziel et al. in patients with chronic hepatitis C. Core peptides 132-145 and 173-186 have also been found to function as CTL determinants in humans. While most CTL determinants that were found in the core protein were previously described, the E1 epitope encountered in this study was novel.

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 cytototoxicity 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.

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, while the E1 312-326 peptide was novel. We selected this peptide, using a chromium release assay. 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.
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 deter-
minant is present in decapeptides 2, 3, and 4, indicating that
the minimal epitope comprises the amino acid sequence
GHRMAWDMMMA.

The minimal determinant identified in Table 3 corresponds
to a highly conserved region of E1 among different geno-
types. 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 preincu-
bat ed with peptide sequences 312-326 from genotypes 1a and
1b. 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 immu-
nized 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-galacto-
sidase (RAdCMV-LacZ) and noninfected cells. Figure 6A
shows that 18Neo cells infected with RAdCMV-CE1, there-
fore 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 com-
plement. 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 comple-
ment or by complement alone (Fig. 6A). This indicates that
the CTL induced by immunization with RAdCMV-CE1 corre-
sponds to cells with the phenotype CD4-CD8+.

Experiments were also performed to map the major histo-
compatibility 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-2b background (P815) and class-I mismatched cells from
an H-2d background (EL-4). Both cell types were preincu-
bated with peptide 312-326 and then analyzed in a cyto-
toxicity assay against lymphocytes from BALB/c mice (H-2d)
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-2d restricted.

To study the kinetics of the specific cytotoxic T-cell activity
-generated by immunization with RAdCMV-CE1, further ex-
periments were performed in which mice were killed between
10 and 100 days, postintraperitoneal injection of the recombi-
nant. We found that spleen cells isolated from mice at 10,
40, and 100 days postimmunization displayed similar cyto-
toxicity when incubated with target cells sensitized with pep-
idate 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 immu-
unization can be sustained for at least 100 days.

**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.41,42,43

Recombinant viruses constitute an efficient means for DNA
transfer to cells both in vitro and in vivo. When transgenes
are placed in these vectors under the drive of a strong pro-
moter such as cytomegalovirus immediate early promoter, as
in this study, it is possible to achieve a high level of expres-
sion of the recombinant proteins. Several reports8,10,44-46 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.

<table>
<thead>
<tr>
<th>Peptide*</th>
<th>Genotype</th>
<th>Ratio 15:1</th>
<th>Ratio 5:1</th>
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<tbody>
<tr>
<td>HVSGHRMAWDMMMA</td>
<td>1a</td>
<td>60</td>
<td>29</td>
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<tr>
<td>–<strong>TT</strong>--------- –</td>
<td>1b</td>
<td>48</td>
<td>26</td>
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<tr>
<td>None</td>
<td></td>
<td>27</td>
<td>11</td>
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* The underlined A was added for synthesis convenience.

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

<table>
<thead>
<tr>
<th>Peptide*</th>
<th>% Lysis</th>
<th>Ratio 50:1</th>
<th>Ratio 1:1</th>
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<tbody>
<tr>
<td>HVSGHRMAWDMMMA</td>
<td>312-326</td>
<td>70</td>
<td>41</td>
</tr>
<tr>
<td>HVSGHRMAWDMA</td>
<td>30</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>VSGHRMAWDMA</td>
<td>57</td>
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<tr>
<td>SGHRMAWDMA</td>
<td>50</td>
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<td></td>
</tr>
<tr>
<td>GHRMAWDMA</td>
<td>45</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>HRMAWDMA</td>
<td>31</td>
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</tr>
<tr>
<td>RMWDMA</td>
<td>25</td>
<td>22</td>
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<td>MAWDMA</td>
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<td>17</td>
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<tr>
<td>None</td>
<td></td>
<td>22</td>
<td>13</td>
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</tbody>
</table>

* The underlined A was added for synthesis convenience.
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., as recognized by liver infiltrating lymphocytes from patients with chronic HCV infection. Peptide 34-47 contains, on prediction, a different epitope which has also been signaled by Cerny et al. and by Battegay et al. as recognized by peripheral blood lymphocytes in 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. have identified core peptide 133-142 as a cytotoxic T-cell determinant in H-2d mice. Minimal epitopes of some of the above described CTL determinants have been characterized. 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, there is also evidence suggesting that immunity to envelope proteins may contribute to viral clearance. 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. 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 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 promotor has succeeded in inducing humoral immunity to HCV.

It is not surprising, therefore, that this strategy has been considered in the development of vaccines against a diversity of viral agents. Because of the safety for humans of disabled recombinant viral vectors, 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. 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 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).
is well known that despite low levels of expression of adenoviral proteins, recombinant defective adenoviruses induce a cyto-
toxic response directed against the vector, which tends to eli-
timate the transduced cells and limits the expression of the
transgenes to a few weeks. 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 ten-
dency. Although, as described previously, CTL against viral
antigens have been found in chronic HCV carriers, the cyto-
toxic T-cell response in this infection appears to be weak or
inefficient. In fact the frequency of CTL precursors against
determinants present in core, envelope proteins, or nonstruc-
tural proteins found in patients with chronic hepatitis C is
between 1/10 and 1/100. 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 hu-
man or cytomegalovirus found in asymptomatic human
immunodeficiency virus or cytomegalovirus-infected patients
which is 1 to 2 x 10^3 and 1 to 5 x 10^3, respectively. It
seems possible, therefore, that procedures aimed at increas-
ing the frequency of CTL precursors in non-infected individu-
als might provide protection against HCV infection. Recombi-
nant 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 deter-
ninants, and another is, as mentioned, inducing a CTL
response of low intensity which, although capable of produc-
cing cell damage, is inefficient to clear the virus. The low
intensity of the CTL response against HCV antigens is pos-
sibly 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 complemen-
tary 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 recomb-
ninant defective adenoviruses, after reducing the viral load
with interferon, might possibly have the beneficial effect of
reducing the risk of posttherapeutic relapse.

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REFERENCES

owski UH. Protection against lethal cytomegalovirus infection by a re-
9. Kast WM, Roux L, Curren J, Blom HJJ, Voorbeu AC, Meelen RH, Kola-
10. Klavinsks LS, Whitton JL, Oldstone MBA. Molecularly engineered vac-
cine which expresses an immunodominant T-cell epitope induces cytotoxic
11. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Improvement of viral immunity in immunodeficient humans by the adoptive trans-
12. Schulz M, Zinkernagel RM, Hengartner H. Peptide induced antiviral pro-
13. Voss E, Dietzschold B, Schaller GM, Herber-Kata E. Synthetic peptide induces long term protection from lethal infection with herpes simplex
14. Yap KL, Ada GL, McKenzie IF. Transfer of specific cytotoxic T lympho-
cyte protects mice inoculated with influenza virus. Nature 1978;273:239-
239.
15. Zinkernagel RM, Doherty PC. MHC restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens deter-
term T cell restriction-specificity function and responsiveness. Adv Imm-
epitope in the hepatitis C virus non-structural RNA polymerase-like pro-
18. Moss B. Vaccinia virus: a tool for research and vaccine development. Sci-
20. Ferrari JB. Solid phase peptide synthesis. I. The synthesis of a tetra-
23. McGrory WJ, Bautista DS, Graham FL. A simple technique for the rescue
of early region 1 mutations into infectious human adenovirus type 5. Virol-
25. Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetra-
solid phase synthesis using N-fluorobenzyloxycarbonyl aminocids on
polyamide supports. Synthesis of substance P and of acetyl carrier protein
65-74 decapeptide. Journal of Chemical Society Perkins Transactions


