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Presence of Effector CD8+ T Cells in Hepatitis C Virus-Exposed Healthy Seronegative Donors

Paola Scognamiglio,* Daniele Accapezzato,† Marco Antonio Casciaro,* Antonella Cacciani,* Marco Artini,* Guglielmo Bruno,* Maria Lucia Chircu,† John Sidney,‡ Scott Southwood,‡ Sergio Abrignani,§ Alessandro Sette,‡ and Vincenzo Barnaba*†

CTL responses against multiple hepatitis C virus (HCV) epitopes were detected in 7 of 29 (24.1%) healthy family members (HFM). These precursor CTL were at very low or undetectable frequencies, as determined by limiting dilution analysis. However, when HCV-specific effector CD8+ T cells, freshly isolated from PBMC of HCV-HFM, were assessed by a sensitive enzyme-linked immunospot assay, their frequencies were severalfold higher than those of precursor CTL. These results indicate that the two assays detect two functionally distinct T cell populations and that the effector cells are not assayed by the 51 Cr-release assay. Furthermore, the combination of cell depletion and enzyme-linked immunospot analyses showed that the effector cells were confined into a CD8+ CD45RO+ CD28− population. The persistence of effector CD8+ T cells specific for both the structural and nonstructural viral proteins in uninfected HCV-HFM, suggest that: 1) an immunological memory is established upon a subclinical infection without any evidence of hepatitis, in a large cohort of HCV-exposed individuals; 2) because these cells required neither restimulation nor the addition of particular cytokines in vitro for differentiating in effectors, they should be capable of prompt HCV-specific effector function in vivo, possibly providing antiviral protection; and 3) the maintenance of effector T cell responses may be sustained by persisting low-level stimulation induced by inapparent infections.

Hepatitis C virus (HCV) is a positive strand RNA virus leading to chronic infection in 50–70% of infected individuals and represents one of the most important causes of hepatocellular carcinoma (1–3). In opposition, some individuals do not develop apparent infection despite persistent exposure to HCV, such as the intrafamilial members of HCV-infected patients (4, 5) or a significant proportion of i.v. drug abusers persistently exposed to the virus through needle sharing (6). Furthermore, the development of cellular immune responses to HCV in some seronegative healthy individuals, who were either sexual partners of HCV-infected patients or laboratory personnel with possible occupational exposure to the virus, has been recently reported (7, 8).

It has been well established that CTLs represent the most important effector arm of the immune system in regards to the elimination of virus-infected cells (9). However, in chronically HCV-infected patients, virus persists despite the fact that CTL specific for multiple HCV epitopes are detectable in peripheral blood and in even higher frequencies in the liver (10–15). This complicates the task of distinguishing clear correlates of protective immunity in HCV infection. The study of the HCV-specific cellular responses in seronegative and healthy individuals, persistently exposed to the virus may help to identify protective HCV epitopes. In this respect, the recent discovery that multiple class I molecules can recognize common peptide motifs (supermotifs) led to the identification of a group of alleles (supertypes) that recognize largely overlapping peptide binding motifs (16–21). Furthermore, peptides with a degenerate capacity of binding multiple class I molecules have been demonstrated to be highly immunogenic in term of induction of CTL responses in different human infections (22–24).

Here, we have studied HCV-specific CTL responses in a cohort of seronegative (for both anti-HCV Abs and HCV-RNA over a 2-year period) healthy family members (HFM), living with at least one patient with chronic HCV infection, and thus potentially exposed to the HCV (HCV-HFM). T cells from these individuals were tested for their capacity to recognize a panel of highly cross-reactive peptides, restricted by the HLA-A2 or -A3 supertype (16–24). Furthermore, these peptides were also all selected from highly conserved regions of the HCV genome. Most importantly, we analyzed the responses of in vivo activated CD8+ T cells capable of rapid effector function within 6 h of contact with class I binding supermotif HCV peptides without previous Ag restimulation or IL-2 addition in vitro (25). To accomplishing the latter, we have used a sensitive ex vivo enzyme-linked immunospot (ELISPOT) assay, allowing the enumeration of individual T cells secreting cytokine molecules that form spots (26, 27), which are generally too few (particularly in noninfected individuals) to be detected by the conventional 3Cr-release cytotoxicity assay (25, 28, 29). Our results illustrate a very sensitive strategy for the identification of the protective cellular immune responses in HCV-HFM.

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Abbreviations used in this paper: HCV, hepatitis C virus; HFM, healthy family members; ELISPOT, enzyme-linked immunospot; LDA, limiting dilution analysis; VV, vaccinia virus; HS, human serum.
Materials and Methods

Study populations

Twenty-nine HCV-HFM (mean age 35; range 18-67; 9 males and 20 females) derived from 19 distinct families with 1 member affected by chronic HCV infection and 20 healthy donors without known close contacts with HCV-infected patients as controls (range age 20-54; 9 males and 11 females) were selected. Moreover, 12 of the HCV-HFM (1 male, 11 females) were sexual partners, 6 sons, 8 daughters, 1 brother, 1 father, and 1 mother of the chronic HCV carriers. All subjects were negative over a 2-year period in repeated tests for the presence of serum anti-HCV Abs (HCV 2.0 ELISA Test System; Ortho Diagnostics, Raritan, NJ) and HCV-RNA, as detected directly from human serum by a reverse transcriptase nested PCR using a commercial kit whose lower limit of detection is 100 copies (High Pure Viral RNA Kit; Boehringer Mannheim, Mannheim, Germany) (30). Both populations were also selected for expression of the HLA-A2 and/or HLA-A3 Ags, as detected by flow cytometry with specific mAbs; the subjects included in the two study groups had clinical or biological signs or history of current or previous liver disease, nor were they exposed to the common risk factors for HCV transmission (i.e., blood transfusion, i.v. drugs or intranasal cocaine, promiscuous sexual relationships, work in clinical setting or laboratories, hospital stay or surgeries, acupuncture, or immunosuppressive drug addition), except the HCV-HFM, who were exposed only to HCV-infected patients. All HCV-HFM and control populations were negative for anti-HIV Abs (ELISA 2; Ortho Diagnostics) and hepatitis B envelope Ag (AUK-3; Sorin Diagnostics S.r.l., Saluggia, Italy). On the contrary, all 19 chronic HCV carriers were seropositive for both HCV-RNA and anti-HCV Abs; none of them were treated with IFN-α or other antiviral or immunosuppressive therapies at the moment of CTL studies in HFM. HCV-RNA was quantitated using a commercial kit whose lower limit of detection is 1000 copies (Amplipore Monitor; Roche, Basel, Switzerland), and the HCV genotype in chronic HCV carriers was also determined.

Synthetic peptides, MHC presentation, and peptide binding affinity assays

Peptides were synthesized in our laboratory, as previously described (31), purified to >95% homogeneity by reverse-phase HPLC, and analyzed by amino acid analysis and/or mass spectrometry analysis. Large epotope libraries were purchased as crude material from Chiron Mimotopes (Chiron, Clayton, Victoria, Australia).

MHC molecules were purified from appropriate EBV-B cell lines by affinity chromatography as previously described (21) upon cell lysate depletion of HLA-B and HLA-C molecules by repeated passage over protein A-Sepharose beads conjugated with the anti-HLA (B,C) Ab B1.23.2 (32) and subsequent HLA-A molecule capture with the anti-HLA (A) B Ab W6/32 (33). Quantitative assays for the binding of peptides to soluble class I molecules based on the inhibition of binding of a radiolabeled standard probe peptide to detergent solubilized MHC molecules have been described previously (20, 31). Class I-specific CTL lines were restimulated onto 96-well plates with whole PBMC plated in 200 μl RPMI/10% HS at dilutions ranging from 12,500 to 200,000 cells/well, in the presence of the peptide (10 μg/ml), which previously resulted in the most stimulation of purified fresh CD8+ T cells in the CTL assay. After 3 days of culture, 50 μM rIL-2 was added, and, after a further 5 days, cultures were restimulated with autologous irradiated peptide-pulsed PBMC. Then, 50 μM rIL-2 was seeded 3-4 days later, and, after a further 5 days, each culture was tested in a specific CTL assay using as targets 51Cr-labeled allogeneic HLA-matched EBV-B cells pulsed or not with the relevant peptide.

Generation of CTL lines

T cell populations were isolated and maintained as previously described (34, 35). Briefly, primary peptide-specific CTL were restimulated onto 96-well round-bottom plates (Falcon Labware, Oxnard, CA) with 10 μg/ml HCV peptide and irradiated (3000 rad) autologous PBMC as APC (5 × 10^5 cell/well). After 5 days, 50 μM human rIL-2 was added, and, after a further 10-12 days, cell growth was detected using an inverted microscope. Growing cultures were then tested for their capacity to mount a specific cytototoxic response to peptide-pulsed 51Cr-labeled target cells. Peptide-specific CTL lines were moved into 24-well plates and were further expanded in rIL-2-containing RPMI/10% FCS.

CTL assay

CTL activity was measured in a 6-h 51Cr-release assay, as previously described (34, 35). 51Cr-labeled EBV-B cell lines, which were previously pulsed or not with peptide (10 μg/ml) for 2 h at 37°C, were used as target cells and were cultured at E:T ratios ranging from 50:1 to 2.5:1.1. The assays were excluded from analysis if the spontaneous release was >25% of maximal release in all assays and were considered positive when peptide-specific lysis was >15% above background (given by culture controls without Ag). In some experiments, EBV-B cells (1.5 × 10^5) were incubated with 1 PFU/1 × 10^5 cells of the different preparations of vaccinia virus (VV)-infected HCV proteins at 4°C for 10 min, washed, and resuspended in 5 ml complete medium and incubated at 37°C 5% CO2 for 12 h before being used as target cells.

ELISPOT assay for detection of Ag-specific effector CD8+ T cells from freshly isolated PBMC and for enumeration of T cell effector frequencies

Ag-specific effectors were detected from freshly purified CD8+ T cells by ELISPOT, as described with minor modifications (26-28). Briefly, 96-well nitrocellulose-backed plates (MAIP S45 10; Millipore, Bedford, MA) were coated with 10 μg/ml capture mouse anti-IFN-γ (Quantigen set; Pharmingen) overnight at 4°C. Plates were then washed six times with PBS/0.05% Tween 20 (Sigma Chemical, St. Louis, MO) before being blocked with 1% BSA in PBS for 30 min at 37°C, and further washed. Irradiated autologous CD8-depleted PBMC as APC, previously pulsed or not with peptide (10 μg/ml) for 2 h, were plated in 100 μl RPMI/10% HS onto precoated plates (2 × 10^5/well) in duplicate, in the presence of different concentrations of autologous purified CD8+ T cells. For the detection of Ag-specific effector frequencies enumerated by ELISPOT, whole PBMC, seeded in duplicate at 2.5 and 1.25 × 10^7/well, were directly incubated in the presence of the relevant peptide. In any case, after 6 h of culture at 37°C C and 5% CO2, plates were extensively washed (10 times) with PBS/0.25% Tween 20, and 100 μl of 1 μg/ml biotinylated secondary anti-IFN-γ mAb (Quantigen set; Pharmingen) was added. After 2 h of incubation at room temperature, plates were washed 6 times and 50 μl avidin-HRP conjugate (Quantigen set; Pharmingen) was added to wells, and the plates were incubated for a further 2 h. Then, plates were washed 6 times, and 50 μl aminothiol carbazole solution (Sigma) was added. After 10-15 min, the colorimetric reaction was stopped by washing with distilled water, and plates were air dried.

In vitro peptide-dependent induction of memory precursor CTL from fresh PBMC and limiting dilution analysis (LDA)

Peptide-driven memory CTL induction was performed at levels of highly purified fresh peripheral CD8+ T cells, using as APC the autologous CD8-depleted PBMC (35, 36). The latter were irradiated (3000 rad) and independently pulsed with the different peptides (10 μg/ml) for 2 h. Pulsed or nonpulsed APC were then plated in 200 μl complete RPMI 1640 supplemented with 10% human serum (RPMI/10% HS) (35, 36) onto 96-well round-bottom plates (20,000 cells) in the presence of 1 × 10^5 cells/well of autologous purified CD8+ T cells (at least four wells for each peptide). After 3 days of culture, 50 μM rIL-2 (Proleukin; Eurocetus, Emeryville, CA) was added, and, after a further 5 days, viable T cells from each culture were tested for specific cytototoxicity. LAs were conducted by setting up replicate microcultures (24 wells for each peptide) in 96-well round-bottom plates with whole PBMC plated in 200 μl RPMI/10% HS at dilutions ranging from 12,500 to 200,000 cells/well, in the presence of the peptide (10 μg/ml), which previously resulted in the most stimulation of purified fresh CD8+ T cells in the CTL assay. After 3 days of culture, 50 μM rIL-2 was added, and, after a further 5 days, cultures were restimulated with autologous irradiated peptide-pulsed PBMC. Then, 50 μM rIL-2 was seeded 3-4 days later, and, after a further 5 days, each culture was tested in a specific CTL assay using as targets 51Cr-labeled allogeneic HLA-matched EBV-B cells pulsed or not with the relevant peptide.
Spots were quantitated using a stereomicroscope (Leica GZ6; Leica, Buffalo, NY) under a magnification of ×16–30. Only spots showing fuzzy borders were enumerated. Responses were considered positive when the number of spots per well with Ag were at least twice that in control wells.

Results

Selection of potential HLA-A2- and HLA-A3-supertype HCV peptides

HCV peptides with totally conserved sequences in at least 11 of the 14 strains (≥79% conservancy) and containing HLA binding motifs were identified, synthesized, and tested for their class I binding capacity, as previously described (16, 17, 19, 23). The motifs were identified, synthesized, and tested for their class I binding affinity, as previously described (16, 17, 19, 23). The binding data is presented as IC50 nanomolar values. Peptides with binding affinity ≤50 nM are classified as “good” binders, and peptides in the 50–500 nM range are classified as “intermediate” binders.

Table I. HLA class I supertype binding capacity of potential HCV epitopes

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LFLNILG GWV</td>
<td>85</td>
<td>NS4</td>
<td>1812</td>
<td>4.2</td>
<td>113.2</td>
<td>3.2</td>
<td>19.5</td>
<td>33.3</td>
<td>5</td>
<td></td>
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<tr>
<td>FL LLADARV</td>
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<td>E2</td>
<td>728</td>
<td>18.2</td>
<td>89.6</td>
<td>149.3</td>
<td>246.7</td>
<td>111.1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>YL VAY QATV</td>
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<td>NS4</td>
<td>1590</td>
<td>20.4</td>
<td>39.1</td>
<td>15.9</td>
<td>82.2</td>
<td>33.3</td>
<td>5</td>
<td></td>
</tr>
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<td>RLVFPDLGV</td>
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<td>NS5</td>
<td>2592</td>
<td>56.2</td>
<td>390.9</td>
<td>10.0</td>
<td>370.0</td>
<td>8000.0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>DLMGY IPLV</td>
<td>79</td>
<td>CORE</td>
<td>132</td>
<td>80.0</td>
<td>477.8</td>
<td>204.1</td>
<td>480.5</td>
<td>12.1</td>
<td>4</td>
<td></td>
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<tr>
<td>WMNR LLIAF A</td>
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<td>NS4</td>
<td>1920</td>
<td>122.0</td>
<td>130.3</td>
<td>3.3</td>
<td>1608.7</td>
<td>400.0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>VLV GVLAA</td>
<td>85</td>
<td>NS4</td>
<td>1666</td>
<td>185.2</td>
<td>330.8</td>
<td>32.3</td>
<td>308.3</td>
<td>3076.9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>HMWNPISG I</td>
<td>92</td>
<td>NS4</td>
<td>1769</td>
<td>15.2</td>
<td>10750.0</td>
<td>76.9</td>
<td>132.1</td>
<td>757.2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>ILAG YGYPL</td>
<td>79</td>
<td>NS4</td>
<td>1851</td>
<td>116.3</td>
<td>143.3</td>
<td>5.0</td>
<td>755.1</td>
<td>888.9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>YLLP RGGPR</td>
<td>92</td>
<td>CORE</td>
<td>35</td>
<td>125.0</td>
<td>6142.9</td>
<td>454.5</td>
<td>415.7</td>
<td>10256.4</td>
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<td></td>
</tr>
<tr>
<td>YLVTRHADV</td>
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<td>NS5</td>
<td>1131</td>
<td>454.5</td>
<td>2047.6</td>
<td>3.6</td>
<td>71.2</td>
<td>3076.9</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

* Peptides with binding affinity ≤50 nM are classified as “good” binders, and peptides in the 50–500 nM range are classified as “intermediate” binders.

Table II. Prevalence of HCV-specific CTL in HFM of chronic HCV carriers distributed in familial categories

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>CTL &gt;20% Lysis to At Least 1 Peptide</th>
<th>CTL &gt;15% Lysis to Multiple Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total HCV-HFM</td>
<td>29</td>
<td>7 (24.1%)</td>
<td>6 (20.7%)</td>
</tr>
<tr>
<td>HLA-A2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23</td>
<td>7 (30.4%)</td>
<td>6 (26.0%)</td>
</tr>
<tr>
<td>HLA-A3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Males</td>
<td>9</td>
<td>3 (33.3%)</td>
<td>3 (33.3%)</td>
</tr>
<tr>
<td>Females</td>
<td>20</td>
<td>4 (20.0%)</td>
<td>3 (15.0%)</td>
</tr>
<tr>
<td>Sexual Partners</td>
<td>12</td>
<td>3 (25.0%)</td>
<td>3 (25.0%)</td>
</tr>
<tr>
<td>Non-Sexual Partners</td>
<td>17</td>
<td>4 (23.5%)</td>
<td>3 (17.6%)</td>
</tr>
<tr>
<td>Male Sexual Partners</td>
<td>1</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Female Sexual Partners</td>
<td>11</td>
<td>3 (25.0%)</td>
<td>3 (25.0%)</td>
</tr>
<tr>
<td>Sons</td>
<td>6</td>
<td>3 (50.0%)</td>
<td>3 (50.0%)</td>
</tr>
<tr>
<td>Daughters</td>
<td>8</td>
<td>1 (12.5%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

* Of the 20 subjects from the control group, 12 were HLA-A2<sup>a</sup> and 8 HLA-A3<sup>a</sup>.

Induction of HCV-specific precursor CTL from PBMC of HCV-HFM

Highly purified CD8<sup>+</sup> T cells derived from PBMC of both HCV-HFM and healthy controls were in vitro stimulated with the HCV peptides and then tested in the CTL assay. All HCV-HFM and control subjects were tested at least two times within 25–30 days. Seven of the HCV-HFM (24.1%) presented reproducible CTL response values against multiple HCV peptides (derived from both the structural and nonstructural viral proteins) in the two consecutive samples taken within 25–30 days (HCV-HFM responders) (Table II and Fig. 1A). Because 23 of the 29 HCV-HFM were HLA-A2<sup>a</sup> (Table II), we were not surprised that all the HCV-HFM responders were confined among the individuals having that allele. In the remaining HCV-HFM, no consistent recall responses were detected against any of the HCV peptides tested. Sporadic CTL responses in some of them to some of the HCV peptides (lysis, <15%) were not confirmed in the following control assays performed 25–30 days later (HCV-HFM nonresponders). These responses are not considered further in our study. No significant correlation was demonstrated between the strength of CTL responses against a given HCV epitope and any of the HCV-HFM familial categories considered (Table II), suggesting that possible inapparent HCV infections may be transmitted through several ways. No significant CTL response against the different HCV peptides studied was shown in the 20 control subjects, who presented always specific cytotoxicity values <5% (data not shown), ruling...
out the possibility that the responses were determined by an in vitro priming (36).

**HCV-specific CTL derived from PBMC of HCV-HFM are class I-restricted and recognize endogenously synthesized HCV epitopes**

Randomly selected HCV-specific CTL lines, derived from HCV-HFM responders, after two rounds of stimulation with autologous peptide-pulsed APC, were tested in MHC-restriction experiments and for their capability to recognize the endogenous forms of the HCV peptides. For HLA-restriction analysis, blocking experiments were performed using culture supernatants of hybridomas BB7.2 and GAP A3 containing anti-HLA-A2 (IgG2b) and anti-HLA-A3 (IgG2a) mAbs, respectively (American Type Culture Collection, Manassas, VA). Hybridoma culture supernatants were added to the 96-well plates of CTL assay at the final dilution of 1:3. Fig. 1B shows that the Ag-specific lysis of CTL lines recognizing HLA-A2 supertype binding HCV peptides was strongly inhibited by anti-HLA-A2, but not by anti-HLA-A3 mAbs, clearly supporting the class I restriction of these responses.

Furthermore, all HCV-specific CTL lines tested (recognizing either NS31131–1139, NS41920–1928, Core35–44, or Core132–140) were able to kill HLA-matched EBV-B cells (HLA*A0201+ JY cell line) (Fig. 2, a–d), but not the HLA*A0301+ HHK EBV-B cell line (Fig. 2, c and d), either when pulsed with the relevant peptide or when infected with recombinant VV expressing the corresponding HCV protein (kindly donated by Dr. M. Houghton, Chiron Corporation, Emeryville, CA). That the HHK EBV-B cells are bona fide target cells is shown by the evidence that, when infected with VV-expressing HCV-Core protein, they were efficiently lysed by a HLA-A3-restricted, Core43–51-specific CTL line derived from the liver of a patient with chronic HCV infection (Fig. 2e). These data suggest that these HCV peptides are endogenously processed and substantiates the class I restriction of the CTL responses.

**HCV peptide-specific effector CD8+ T cells can be isolated from PBMC of HCV-HFM and their frequency is higher than that of the HCV-specific precursor CD8+ T cells detected by LDA**

Highly purified CD8+ T cells, freshly isolated from PBMC of HCV-HFM or control subjects, were tested by ELISPOT assay for the capacity to form spots representing IFN-γ produced by individual cells (IFN-γ spots) in response to class I-restricted HCV
epitopes. Four of the seven HCV-HFM responders reproducibly showed, in both the two consecutive ELISPOT assays performed within 1 mo from each other, peripheral CD8+ T cells with prompt effector function within h of TCR occupancy with at least one HCV peptide (Fig. 3, a–d). Recall responses from the same responders were previously demonstrated for the same peptides. However, the finding that CD8+ T cells, derived from the three ELISPOT-negative HCV-HFM responders, synthesized IFN-γ following two rounds of Ag-stimulation in vitro, rules out the possibility that they were defective in producing that cytokine. Conversely, no response was evident in wells where fresh CD8+ T cells plus APC were incubated with irrelevant HLA-A2 or -A3 binding HIV peptides (gp120121–129 or gag 174 –182, respectively) or without any peptide (Fig. 3), or in wells where irradiated or not CD8-depleted cells, as APC, were incubated with relevant HCV peptides without responder cells (not shown). Moreover, CD8+ T cells derived from 10 healthy controls tested did not yield any IFN-γ spots in response to any of the HCV peptides taken in consideration (not shown). We also tested fresh CD8+ T cells following immunomagnetic depletion of either CD45RA+ or CD28+ cells (37). Fig. 4 shows that the depletion of the CD45RA+ cells significantly decreased the number of IFN-γ-producing cells upon Ag-dependent TCR ligation. On the contrary, the depletion of the CD45RA+ or the CD28+ subsets did not affect the formation of IFN-γ spots, but rather increased their number. These results suggest that HCV-specific effector T cells are confined in a subset CD45RA+CD28+ and that they could derive by the restimulation of memory T cell precursors (CD45RA+CD28+). In an attempt to define the phenotype of the HCV-specific effector CD8+ T cells, we studied the effector function of CD8+ T cells following immunomagnetic depletion of either CD45RO+ or CD45RA+ or CD28+ cells (37). Fig. 4 shows that the depletion of the CD45RO+ cells significantly increased the number of IFN-γ-producing cells upon Ag-dependent TCR ligation. On the contrary, the depletion of the CD45RA+ or the CD28+ subsets did not affect the formation of IFN-γ spots, but rather increased their number. These results suggest that HCV-specific effector T cells are confined in a subset CD45RO+CD28+ and that they could derive by the restimulation of memory T cell precursors (CD45RO+CD28+) (37–39).

Discussion

The data in this paper make two main points. First, healthy subjects persistently exposed to HCV-infected patients develop CD8+ T cells (25–29). Thus, we compared the HCV-specific effector CD8+ T cell frequency detected by ELISPOT with that of the corresponding precursor CTL detected by LDA and found that the former was severalfold higher than the latter in all individuals tested (Table III). This is consistent with recent data demonstrating a comparable sensitivity of the ELISPOT assay and the counting of Ag-specific T cells by MHC-peptide complex tetramers and that both were more sensitive than the LDA assay (25, 28, 29).
T cell responses against not only the structural but also nonstructural HCV proteins, whose production is absolutely dependent on the viral replication. Thus, in contrast with the current epidemiological data, a large fraction of healthy individuals may undergo a subclinical HCV infection and clear the virus, possibly upon the development of a protective immunity. At the light of this assumption, the evidence that no correlation was evident between the HCV-specific CTL responses and any of the HCV-HFM familial categories (see Table II) suggests that intrafamilial HCV transmission may spread via several inapparent ways. Second, HCV-specific CD8\(^+\) T cells displaying prompt effector functions in vitro circulate at a low frequency in the peripheral blood of HCV-HFM. Importantly, however, these effector CD8\(^+\) T cells were detected at severalfold higher frequency than CTL tested by LDA. This may be determined by the fact that LDA detects only precursors requiring proliferation upon TCR occupancy for becoming effectors, but ignore effector T cells, which lack this proliferative potential and, because highly activated, are sensitive to apoptosis upon Ag restimulation (39). In addition, it is also possible that many HCV-specific CD8\(^+\) T cells are able to secrete IFN-\(\gamma\), but only a subset of them acquire cytotoxicity function. However, various evidences suggest that virus-specific CD8\(^+\) T cells secreting IFN-\(\gamma\) have a...
Table III. HCV-specific effector T cell frequencies enumerated by IFN-γ ELISPOT and corresponding precursor frequencies by LDA in HFM of chronically HCV-infected patients

<table>
<thead>
<tr>
<th>HCV-HFM</th>
<th>HCV Epitope</th>
<th>HLA Class I Restriction</th>
<th>Effector Frequency in PBMC by ELISPOT</th>
<th>Precursor Frequency in PBMC by LDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>R.N.</td>
<td>Core31–43</td>
<td>A*0201</td>
<td>1/12,453</td>
<td>1/345,000</td>
</tr>
<tr>
<td>M.C.</td>
<td>NS3131–339</td>
<td>A*0201</td>
<td>1/13,675</td>
<td>1/450,000</td>
</tr>
<tr>
<td>S.G.</td>
<td>NS4166–74</td>
<td>A*0201</td>
<td>1/12,500</td>
<td>1/225,550</td>
</tr>
<tr>
<td>F.L.</td>
<td>NS3/NS4172–174</td>
<td>A*0201</td>
<td>1/15,100</td>
<td>1/669,200</td>
</tr>
<tr>
<td>C.V.</td>
<td>NS3131–339</td>
<td>A*0201</td>
<td>1/10,395</td>
<td>1/738,000</td>
</tr>
<tr>
<td>R.A.M.</td>
<td>NS31096–1104</td>
<td>A*0301</td>
<td>1/100,000</td>
<td>Undetectable</td>
</tr>
<tr>
<td>G.M.</td>
<td>Peptide pool</td>
<td>A*0301</td>
<td>1/35,714</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* LDA and ELISPOT were performed as described in Materials and Methods. For LDA, wells were considered positive when specific lysis was >15% above background.

**FIGURE 4.** The majority of HCV-specific effector CD8 T cells are confined in a CD45RO CD28 population. Fresh PBMC from two HCV-HFM responders (a and b) were depleted of either CD45RO or CD45RA or CD28 cells by immunomagnetic separation as described in Materials and Methods. Then, cells were tested in ELISPOT for their capability to form IFN-γ spots within 6 h of contact with HLA-A2 binding peptide pool. FACS analysis demonstrated 65–70% depletion of mAb-stained cells from PBMC. Data represent means of duplicate determinations.

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direct effect on virus replication in infected cells and rather seem to be more efficient for protection than perforin- or Fas-mediated lysis in some viral infections (40–42).

Other studies have shown memory CTL responses to HCV in seronegative persons (7, 8), but our is the first report describing the presence of HCV-specific effector CD8 T cells in healthy aviremic subjects persistently exposed to HCV-infected patients. Although our data cannot demonstrate a direct relationship between effector T cells and HCV clearance, the finding that these cells required neither restimulation nor the addition of particular cytokines in vitro for differentiating in effectors indicates that they should be capable of prompt HCV-specific effector function in vivo, possibly providing antiviral protection. The protective potential of the effector CD8 T cells has been recently supported in different models of viral infection, demonstrating a clear inverse correlation between effector CTL frequency and viral load (25, 28, 29, 43, 44). The presence of effector CD8 T cells in HCV-HFM may in part explain the low level of intrafamilial HCV infection and possibly the inefficient HCV infection in the large cohort of individuals who are repeatedly exposed to HCV (4–8, 45).

Recently, in the attempt to separate phenotypically memory and effector human CD8 T cells, it has been elegantly demonstrated that the former are CD45RO CD27 CD28 + exerting effector functions only upon in vitro proliferation and the latter are CD45RA CD27 CD28 + and perform effector functions without previous in vitro stimulation (37). The finding that our effector cells were confined in a subset CD45RO CD28 - resembling only in part the effector CD45RA CD27 CD28 + cell population described by Hamann et al. (37), suggests that effector cells may belong to an heterogeneous population, i.e., CD45RA CD28 - cells derived by the priming of naive CD45RA CD28 + T cells and CD45RO CD28 - cells generated by the restimulation of memory T cell precursors (CD45RO CD28 +). This possibility may reconcile the above mentioned data (37), our data, as well as those of Lalvani et al. showing that influenza-specific effectors are CD45RO (25). Thus, it is tempting to presume that the majority of HCV-specific effector CD8 T cells, identified in our study, derive by the continuous activation of memory resting T cells. The lack of CD28 molecules further supports the effector state of this subset (38), implying that these cells are insensitive to costimulation by APC and thus are possibly prone to death once they performed their functions (39).

What maintains the persistence of HCV-specific effector CD8 T cells in HCV-HFM is unclear. We hypothesize that the continuous exposure to chronically infected patients may provide a persistent and inapparent source of HCV infection, as it has been proposed for HIV-exposed seronegative individuals (46–50). This persisting low-level stimulation may on the one hand induce effector T cell responses, and on the other hand expand the memory precursor T cell pool, which in turn would be persistently alerted to supply recurrent waves of cells becoming effectors (32, 51–54). The finding that HCV-specific effector T cells were not detected in all HCV-HFM with recall responses to HCV is consistent with the possibility of a recent, inapparent HCV infection in those individuals developing effector cells. Alternatively, effector T cells could persist at low frequency long after the recovery from an inapparent HCV infection, as recently demonstrated in healthy individuals previously exposed to influenza virus (25). On the other hand, we cannot completely exclude that effector T cells are sustained by a minimal viral load hidden in liver or in other unknown privileged tissues, or by cross-reactive Ags. In any case, the effector CD8 T cells may allow the prevention of the viral spread in a significant percentage of infected individuals (1, 2).
We thank Marino Paroli for critical reading of the manuscript.

immunity against most, if not all, HCV isolates. Alternatively, the prompt CTL priming may provide an important rationale for the design of a prophylactic regimen for HCV.

Altogether these data suggest that the establishment of an immunity vs an immunopathology state against HCV might depend on the prompt vs the delayed rising of the host-specific T cell response. Therefore, an immunity state could take place only in that minority of individuals having pre-existing HCV-specific effector T cells, i.e., induced by infected HCV, as it may occur in our HCV-HFM. Conversely, the majority of infected individuals lacking these effector cells will mount a tardy CTL response (10–15), which will be unable to clear the virus, with subsequent establishment of chronic immunopathology.

Finally, the study of HCV-specific effector T cells in HCV-HFM may provide an important rationale for the design of a prophylactic/therapeutic vaccine-priming Ag-specific CTL responses. In this respect, it should be noted that all epitopes used in the present study are highly conserved and should therefore induce effective immunity against most, if not all, HCV isolates.

Acknowledgments

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References


