

# Cellular Immunity to Hepatitis C Virus Core Protein and the Response to Interferon in Patients With Chronic Hepatitis C

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To investigate the involvement of T-cell response against hepatitis C virus (HCV) antigens in viral clearance after interferon therapy, we measured interleukin-2 (IL-2) production by peripheral mononuclear cells in response to HCV core in patients with chronic hepatitis C. In a cohort of 43 patients, we investigated the frequency of circulating core-specific T-helper (Th) cell precursors by the limiting-dilution assay, and in a second cohort of 60 patients, we analyzed the response to specific core epitopes using 52 synthetic 15-mer overlapping peptides. We observed that the frequency of core-specific Th cell precursors was significantly higher in patients with sustained biochemical and virological response (SR) after interferon (IFN) therapy (median, 1/55,736) than in untreated patients (1/274,023) or that in patients who remained viremic after completion of the treatment—nonresponders (NR) plus transient responders (TR) (1/1,909,972). Patients who failed to respond to IFN (NR) and those who relapsed after IFN discontinuation (TR) had a similarly low number of precursors. The number of core peptides recognized by SR, TR, NR, UT, and healthy controls was  $8.2 \pm 1.5$ ,  $6.5 \pm 1.2$ ,  $2.0 \pm 0.5$ ,  $2.7 \pm 0.9$ , and  $0.3 \pm 0.2$ , respectively. In SR, the intensity of the proliferative response to core peptides as estimated by the summation of stimulation indexes ( $\Sigma$ SI) was significantly higher than in NR and than in UT, but not different from that of TR. Our results indicate that both expansion of HCV-specific Th cell precursors and Th cell recognition of multiple core epitopes seem to be important in the elimination of HCV after IFN therapy. (HEPATOLOGY 1998;28:815-822.)

Hepatitis C virus (HCV) is a small enveloped RNA virus that is now recognized as the major agent of chronic hepatitis and liver disease worldwide.<sup>1-3</sup> More than 70% of those who contract HCV develop chronic infection and hepatitis, and a

significant proportion of them progress to cirrhosis and eventually to hepatocellular carcinoma.<sup>4,5</sup> Interferon alpha (IFN- $\alpha$ ) induces normalization of serum transaminases in 30% to 40% of patients with chronic hepatitis C, but half of them relapse after discontinuation of the therapy.<sup>6</sup> Thus, only around 15% to 20% of cases experience sustained biochemical and virological response to treatment.<sup>7</sup> IFN- $\alpha$  has both direct antiviral activity and immunomodulatory properties,<sup>8</sup> and these two effects may contribute to the elimination of the virus. However, little is known about the mechanisms that determine that some patients respond to the therapy with clearance of the virus and resolution of the disease, while in others, treatment fails to eliminate the infection.

The quasispecies nature of HCV and the antigenic drift of this virus have been proposed as mechanisms for viral persistence.<sup>9</sup> Chronic HCV infection occurs in the presence of circulating antibodies against structural and nonstructural proteins, indicating that humoral immunity is not protective in this infection, possibly because of emergence of scape strains within the quasispecies of the virus.<sup>9,10</sup> Although cellular immunity plays a critical role in the control of viral infections, its contribution to the resolution of hepatitis C remains to be elucidated. In particular, little is known about whether the antiviral effect of IFN- $\alpha$  in patients with chronic HCV infection may depend on the induction of an efficient T-cell immune response against viral antigens.

T-helper (Th) cells are crucial in the orchestration of cellular immunity against intracellular pathogens. These cells recognize processed viral antigenic peptides bound to HLA class II molecules on the membrane of antigen-presenting cells. Th lymphocytes play an essential role in the activation of cytotoxic T lymphocytes,<sup>11-13</sup> which, in addition to mediating the elimination of virus-infected cells, participate together with Th lymphocytes in the production of cytokines that can inhibit viral replication.<sup>14</sup> Because T-cell help seems to play a role in the spontaneous resolution of HCV infection,<sup>15,16</sup> we hypothesized that Th responses might be important for viral clearance following IFN- $\alpha$  therapy. Thus, we studied Th cell reactivity against HCV core protein (a protein highly conserved among different isolates) and core peptides in different groups of patients with chronic hepatitis C according to their response to IFN- $\alpha$ .

## PATIENTS AND METHODS

### Patients

For this study, we selected 80 patients with chronic HCV infection. All of them were anti-HCV-positive and had raised transaminase levels for more than 6 months before starting IFN- $\alpha$  treatment. Patients were classified into the following groups: pa-

Abbreviations: HCV, hepatitis C virus; IFN, interferon; Th, T helper; UT, untreated patients; SR, sustained responders; NR, nonresponders; TR, transient responders; LDA, limiting-dilution analysis; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagutinin; SI, stimulation index; PCR, polymerase chain reaction.

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tients who had not received IFN- $\alpha$  therapy (untreated patients [UT]); patients with biochemical and virological response (normal serum transaminases and negative serum HCV RNA) lasting for at least 6 months after stopping IFN- $\alpha$  therapy (sustained response [SR]); patients who failed to normalize serum transaminases during treatment with IFN- $\alpha$  and remained viremic (nonresponders [NR]); and patients with normal serum transaminases at the end of the treatment but who relapsed after discontinuation of the therapy (transient response [TR]). Two cohorts of patients were studied.

**Cohort A: Limiting-Dilution Analysis.** This cohort comprised 43 patients subdivided into three groups: 12 with sustained biochemical and virological response to IFN- $\alpha$  therapy, 11 patients who failed to exhibit sustained response to therapy (5 were nonresponders and 6 transient responders), and 20 untreated patients. Thus, among treated patients, we have distinguished two main groups: those who cleared the virus after receiving IFN- $\alpha$  therapy (SR) and those who remained viremic after completion of the treatment (NR + TR). Patients were studied  $36.7 \pm 16.9$  and  $27.8 \pm 15.1$  months after the end of treatment in groups SR and NR + TR, respectively. A group of 6 anti-HCV-negative healthy donors (2 females and 4 males; ages 26 to 33 years; mean age,  $27.8 \pm 2.8$  years) was also included as a control group.

**Cohort B: Characterization of Immune Response Against Synthetic HCV Core Peptides.** This cohort comprised 60 patients (23 of them also included in the first cohort) subdivided into four groups: 15 SR, 12 TR, 10 NR, and 23 UT. A group of 7 HCV-seronegative healthy donors (2 females, 5 males; ages 27 to 55 years; mean age,  $33.3 \pm 3.7$  years) was also studied as a control group (C). Patients were investigated  $7.2 \pm 5.4$ ,  $9.4 \pm 6.9$ , and  $8.3 \pm 5.8$  months after completion of IFN- $\alpha$  treatment in groups SR, TR, and NR, respectively.

Table 1 summarizes clinical, virological, and histological characteristics of the different groups of patients from the two cohorts. As previously reported by others,<sup>17</sup> we have found that SRs have, at baseline, significantly lower viral load than NRs ( $P < .01$ ).

## Methods

**Peptide Synthesis.** Peptides were synthesized by the solid-phase method of Merrifield<sup>18</sup> using the Fmoc alternative,<sup>19</sup> utilizing a manual multiple solid-phase peptide synthesizer.<sup>20</sup> The ninhydrin test of Kaiser was used to monitor every step.<sup>21</sup> At the end of the synthesis, peptides were cleaved and deprotected, and washed six times with diethyl ether. They were lyophilized and used without further purification.

**Limiting-Dilution Analysis.** The limiting-dilution analysis (LDA), used to determine frequencies of immunocompetent cells possessing a defined function, requires that all conditions necessary for the detection of a single immunocompetent cell be nonlimiting. A positive response in a culture will then minimally depend on the presence of a single immunocompetent cell (single-hit model). Under our culture conditions, we assume that a single stimulated helper T cell is able to produce enough interleukin-2 (IL-2) to support the growing of a cell line dependent on this cytokine (CTLL cells) at least three times over a negative control. Then, applying a mathematical algorithm based on the Poisson probability theory, we quantified the frequency of Th cell precursors in a population of peripheral blood mononuclear cells (PBMC).

PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque centrifugation and washed three times with saline. Four different concentrations of PBMC ( $2 \times 10^5$ ,  $1 \times 10^5$ ,  $5 \times 10^4$ , and  $2.5 \times 10^4$ ) were placed in 20 replica cultures (for each dilution) in RPMI-1640 medium containing L-glutamine (2 mmol/L), gentamicin (10  $\mu$ g/mL), penicillin (50 U/mL), streptomycin (50  $\mu$ g/mL), and HEPES (5 mmol/L), supplemented with 10% heat-inactivated human AB serum, in the presence of 1  $\mu$ g/mL of recombinant HCV-core protein genotype 1b (a generous gift from Drs. B. Rodgers and B. Clarke, Glaxo-Wellcome, England), complemented with graded numbers of autologous irradiated PBMC (3,000 rads) to give a total number of  $2 \times 10^5$  cells per well (96-well flat-bottomed plates). Ten micrograms per milliliter of an anti-IL-2 receptor monoclonal antibody (BT563, Biotest Pharma, Dreieich, Germany) was added to the cultures according to previously described protocols.<sup>22,23</sup> Mitogen stimulation of cells (positive control) was performed by incubation with phytohemagglutinin (PHA) (1/200 final dilution; Gibco BRL, Grand Island, NY). After 7 days of culture at 37°C and 5% CO<sub>2</sub>, 50  $\mu$ L of supernatant was harvested from cultures and stored at -20°C. The presence of IL-2 on the supernatants was assessed by examining the ability to support the growth of the IL-2-dependent CTLL-2 mouse cell line. CTLL cells were resuspended in RPMI-1640 with 10% fetal calf serum, antibiotics, and 2-mercaptoethanol ( $5 \times 10^{-5}$  mol/L), and were plated ( $5 \times 10^3$  cells per well) in a 96-well flat-bottomed plate with the supernatant to be assayed (25% vol/vol). After 24 hours of culture, the stimulated CTLL cells were pulsed with 1  $\mu$ Ci per well of [<sup>3</sup>H]thymidine for 18 hours, and thymidine incorporation was determined by liquid scintillation (Topcount, Packard, Meridan, CT). Positive responses

TABLE 1. Clinical, Virological, and Biochemical Characteristics of Patients With Chronic Hepatitis C

Response to IFN	Cohort A				Cohort B			
	UT	NR	TR	SR	UT	NR	TR	SR
Number of patients	20	5	6	12	23	10	12	15
Age (yr)	$35.3 \pm 11$	$50.8 \pm 19.5$	$50.8 \pm 14.5$	$44.7 \pm 14$	$53.8 \pm 5.1$	$47.5 \pm 3.9$	$40.3 \pm 4$	$39.5 \pm 11.1$
Sex (male/female)	14/6	2/3	2/4	9/3	9/14	5/5	7/5	12/3
Viral load ( $\times 10^6$ )*	$82.5 \pm 156$	$59.3 \pm 93.9$	$36.8 \pm 39.5$	$12.2 \pm 2.8$	$78.6 \pm 162.5$	$170.4 \pm 198.8$	$30.7 \pm 31.4$	$13.9 \pm 26.7$
ALT	$62.8 \pm 46.4$	$124 \pm 137.1$	$113.2 \pm 65.9$	$15.1 \pm 4.8$	$92.5 \pm 85.1$	$74.9 \pm 28.5$	$86.7 \pm 93.4$	$13.8 \pm 3.7$
Genotype								
1a	4	1		1	4	1		1
1b	10	3	6	3	14	7	10	2
2a								
2b								
3	4	1†		6	2	1	1	10
4	2†				2			
nt				2	1	1	1	2
Histology cirrhosis/noncirrhosis	2/17§	1/5	0/6	1/11	2/21	1/9	0/12	0/15

Abbreviations: nt, not tested; ALT, alanine transaminase.

\*Viral load measured before IFN- $\alpha$  treatment.

†One of these patients was coinfecting with genotype 1b.

‡Patient coinfecting with genotype 4.

§Liver biopsy was available from 19 patients in group UT from cohort A.

for the individual wells were considered when the stimulation index (SI) (ratio of the response to HCV-core protein over mean response in absence of protein) was above three times the negative control. Th cell precursor frequencies were calculated on the regression curve by interpolating the number of responder cells required to give 37% negative cultures. The correlation coefficient was greater than 0.9 in all cases. Only those experiments were considered for which the data fit the single-hit model<sup>24</sup> (evaluation according to  $\chi^2$ ).

**Production of IL-2 by PBMC in Response to Synthetic HCV-Core Peptides.** PBMC were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated human AB serum and 1% antibiotics, plated at  $2 \times 10^5$  cells per well, and cultured in triplicate in 96-well flat-bottomed plates with 10  $\mu\text{g}/\text{mL}$  of an anti-IL-2 receptor monoclonal antibody (BT563, Biotest Pharma) in the presence or absence of synthetic peptides at 25  $\mu\text{g}/\text{mL}$  or PHA (1/200 final dilution; Gibco BRL) as a positive control. After 7 days of culture, supernatants were harvested and stored at  $-20^\circ\text{C}$ . IL-2 production was assessed by examining the ability to support the growth of an IL-2-dependent CTLL mouse cell line as described above. Data are reported as the SI, which is the ratio of mean response to peptide over mean response to a negative control. A positive result was considered when  $\text{SI} \geq 5$ . This limit was taken to eliminate unspecific responses (as observed in healthy controls).

**Serum HCV RNA, HCV Genotypes, and Viral Load.** Serum HCV RNA was performed by the polymerase chain reaction (PCR) as previously described.<sup>25,26</sup> Genotyping was performed using a hybridization technique with specific probes for HCV genotypes 1a, 1b, 2a, 2b, and 3a according to Simmonds et al.,<sup>27</sup> with the amplified HCV core region by nested PCR as described by Viazov et al.<sup>28</sup> with the following technical modifications: primers for the second PCR were 5'-digoxigenin-labeled, and hybridization was detected using an anti-digoxigenin peroxidase-labeled antibody (Boehringer-Mannheim, Mannheim, Germany). HCV RNA was quantified by a competitive PCR technique as previously reported.<sup>25,26</sup>

**HLA Typing.** HLA-DR genotyping was performed by reverse hybridization using the Standard INNO-LIPA HLA DRB kit (Innogenetics, Ghent, Belgium). Assays were performed according to the manufacturer's instructions.

**Statistical Analysis.** Results are presented as means  $\pm$  SEM unless otherwise indicated. The Shapiro-Wilks normality test was used to assess the normality of continuous data. Some variables were logarithmically transformed for statistical analysis. For normally distributed variables, one-way ANOVA followed by Student-Newman-Keuls multiple comparison tests were used to analyze differences between groups of patients. Non-normally distributed variables were analyzed by the Kruskal-Wallis test, followed by Mann-Whitney's  $U$  as a multiple comparison test. Association between continuous variables was studied with Spearman's correlation coefficient. Fisher's Exact test was used to analyze differences between two percentages. Forward stepwise logistic regression analysis was used to assess the independent association of some peptides to sustained response to IFN- $\alpha$  therapy and to calculate their adjusted odds ratios. All statistical analyses were performed with SPSS v 6.0 for Windows package. All  $P$  values were two-tailed.

## RESULTS

**Frequency of HCV Core-Specific Th Cell Precursors.** Frequencies of Th precursors recognizing HCV core protein were calculated by LDA of PBMC from healthy controls and HCV-infected patients. PBMC from all subjects were tested with PHA (as a positive control of the assay), and in all cases, a  $\text{SI} > 35$  was obtained, indicating preserved T-cell reactivity.

The frequency of core-specific Th precursors is significantly higher in SR (median: 1 cell in 55,736 PBMC) than in patients who remained viremic after completion of IFN therapy (NR + TR; 1/1,909,972;  $P < .0001$ ) with very little overlap between the two groups (Fig. 1). Thus, all SR

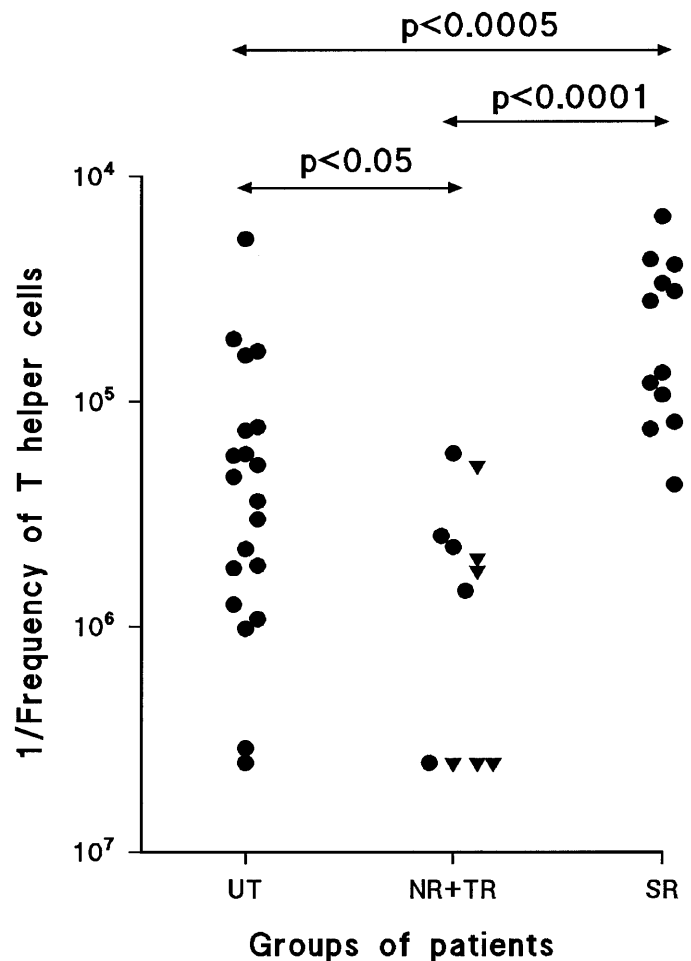


FIG. 1. Frequency of Th cell precursors (IL-2-producing cells) recognizing HCV core protein in the PBMC population from different groups of patients with chronic hepatitis C classified according to the response to IFN- $\alpha$  treatment. Calculation of the frequency of Th precursors was performed by LDA. UT, untreated patients; NR, patients who failed to respond to interferon; TR, transient responders, patients who showed a complete response to IFN but relapsed upon IFN- $\alpha$  withdrawal; SR, patients with sustained response to interferon. ( $\blacktriangledown$ ), correspond to TR patients.

patients, except one, had precursor frequencies above 1 in  $1.5 \times 10^5$  cells, while all patients who failed to exhibit sustained response to treatment had frequencies below this value. The abundance of circulating core-specific Th precursors was similar in NR and TR (see Fig. 1), and the frequency value in these two groups was not significantly higher than that found in HCV-seronegative healthy controls (median: 1/650,000). UT patients exhibited a wide range of values with a median (1/274,023) that was significantly lower than in SR ( $P < .0005$ ), but significantly higher than NR + TR ( $P < .05$ ). Figure 2 shows the analysis of core-specific Th precursors frequency in two representative cases, one SR and one NR. Results are represented as a semilogarithmic plot of responder cell input per well against the percentage of negative wells, with the 95% confidence intervals. As can be seen in the SR case, the percentage of negative wells decreases sharply as the number of cells per well increases, while there is little decrease of negative wells with increasing cell input in the NR case.

It is interesting to note that, despite the fact that SR patients were studied  $36.7 \pm 16.9$  months after viral clearance following IFN- $\alpha$  treatment, they exhibited strong Th

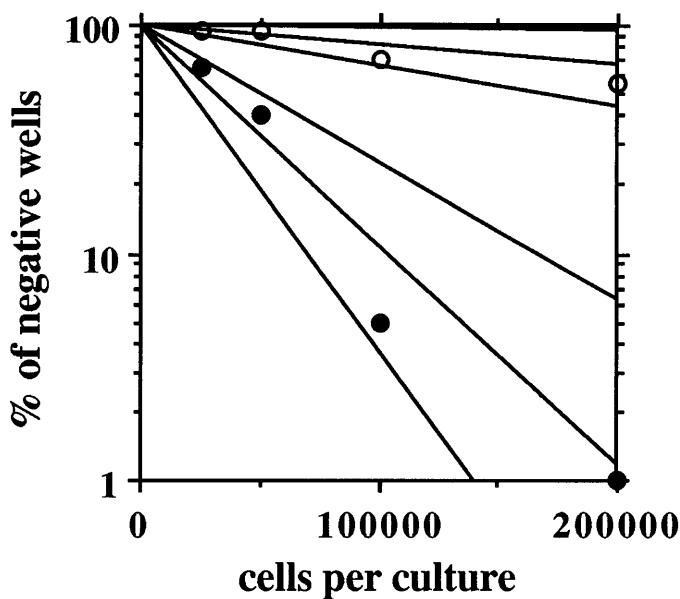


FIG. 2. Comparison of Th cell precursors in two representative cases of SR and NR patients using LDA. The results are shown as a semilogarithmic plot of responder cell input per well against the percentage of negative wells. 95% confidence intervals are represented by dotted lines. (●), SR; (○), NR.

responses against HCV core antigen. Particularly, patient SR8 presented a very high frequency of Th cell precursors (1 per 15,126 PBMC) more than 6 years after completion of therapy.

#### Production of IL-2 in Response to Synthetic HCV Core Peptides.

To analyze Th response to specific HCV core protein epitopes, we synthesized 26 15-mer overlapping peptides

(10-amino acid overlap) from conserved regions of this protein, corresponding to genotypes 1a and 1b, as well as 26 other peptides from variable regions (Table 2). Results of IL-2 production by PBMC in response to HCV core synthetic peptides in the different groups of patients and controls are presented in Fig. 3. As shown, the strongest Th response against HCV-core peptides is found in the SR group, followed by patients with transient response to IFN- $\alpha$  and untreated patients. The response is particularly poor in NR, being very similar to that found in healthy controls (results in these subjects are not shown). Thus, the mean number of HCV-core peptides recognized by individuals from groups SR, TR, UT, NR, and controls was  $8.2 \pm 1.5$ ,  $6.5 \pm 1.2$ ,  $2.7 \pm 0.9$ ,  $2.0 \pm 0.5$ , and  $0.3 \pm 0.2$ , respectively, the values in the first three groups being significantly higher than in controls ( $P < .05$ ). The mean number of peptides recognized by SR and TR patients was significantly higher than in UT and NR ( $P < .05$ ). When we calculated the  $\Sigma$ SI of peptides eliciting a positive response ( $SI \geq 5$ ) in each patient from each group (Fig. 4) as a reflection of the intensity of T-cell activation induced by HCV core epitopes, we found that the median value was also higher in SR and TR patients than in NR, UT, and controls. Thus, both diversity (measured by the number of peptides recognized) and intensity (measured by the value of SI) of Th response appear to be associated with response to therapy and clearance of the virus. As shown in Fig. 3, all patients exhibited a vigorous response to PHA, indicating that overall T-cell function was preserved in all groups.

When we analyzed the IL-2 response against individual peptides, we observed that positive responses against peptides c99-112 and c146-159 were present more frequently among SR patients than among patients with persisting

TABLE 2. Synthetic Peptides From HCV Core Protein

Peptide Name	Position (genotype)	Sequence	Peptide Name	Position (genotype)	Sequence
c1	1-14 (1a)	MSTIPK <b>P</b> QRKTKR <b>N</b> A	c27	81-94 (1a)	YWPWLYGNEGCGW <b>A</b>
c2	1-14 (1b)	MST <b>N</b> PK <b>E</b> FRKTKR <b>N</b> A	c28	81-94 (1b)	YWPWLYGNEG <b>M</b> GW <b>A</b>
c3	6-19 (1a)	K <b>P</b> QRKTKRNTNRR <b>P</b> A	c29	85-98 (1a)	LYGNEGCGWAGWLL <b>A</b>
c4	6-19 (1b)	K <b>E</b> FRKTKRNTLRR <b>P</b> A	c30	85-98 (1b)	LYGNEG <b>M</b> GWAGWLL <b>A</b>
c5	11-24 (1a)	TKRNTNRRPQDV <b>K</b> F <b>A</b>	c31	90-103 (1a)	GCGWAGWLLSPRGS <b>A</b>
c6	11-24 (1b)	TKRNTLRRPQDV <b>R</b> F <b>A</b>	c32	90-103 (1b)	<b>G</b> MGWAGWLLSPRGS <b>A</b>
c7	15-28 (1a)	TNRRPQDV <b>R</b> FPGGG <b>A</b>	c33	95-108	GWLLSPRGRSPSWG <b>A</b>
c8	15-28 (1b)	T <b>L</b> RRPQDV <b>R</b> FPGGG <b>A</b>	c34	99-112	SPRGRSPSWGPTDP <b>A</b>
c9	20-33 (1a)	QDV <b>K</b> FPGGGQIVGG <b>A</b>	c35	104-117	RPSWGPTDPRRR <b>S</b> R <b>A</b>
c10	20-33 (1b)	QDV <b>R</b> FPGGGQIVGG <b>A</b>	c36	109-122	PTDPRRRSRNLGK <b>V</b> A
c11	25-38	PGGGQIVGGVYLL <b>P</b> A	c37	113-126	RRRSRNLGKVIDT <b>L</b> A
c12	29-42	QIVGGVYLLPRR <b>G</b> P <b>A</b>	c38	118-131	NLGKVIDTLTCG <b>F</b> A
c13	34-47	VYLLPRR <b>G</b> PR <b>L</b> GV <b>R</b> A	c39	123-136	IDTLTCGFADLMG <b>Y</b> A
c14	39-52	RR <b>G</b> PR <b>L</b> GV <b>R</b> AT <b>R</b> K <b>T</b> A	c40	127-140	TCGFADLMG <b>Y</b> I <b>P</b> L <b>V</b> A
c15	43-56	RL <b>G</b> V <b>R</b> AT <b>R</b> K <b>T</b> S <b>E</b> R <b>S</b> A	c41	132-145	DLMG <b>Y</b> I <b>P</b> LVGAP <b>L</b> G <b>A</b>
c16	48-61	AT <b>R</b> K <b>T</b> S <b>E</b> R <b>S</b> Q <b>P</b> R <b>G</b> R <b>A</b>	c42	137-150	I <b>P</b> LVGAP <b>L</b> GGA <b>R</b> A
c17	53-66	S <b>E</b> R <b>S</b> Q <b>P</b> R <b>G</b> R <b>R</b> Q <b>P</b> I <b>P</b> A	c43	141-154	GAP <b>L</b> GG <b>A</b> AR <b>A</b> L <b>H</b> G <b>A</b>
c18	57-70 (1a)	Q <b>P</b> R <b>G</b> R <b>R</b> Q <b>P</b> I <b>P</b> K <b>V</b> R <b>R</b> A	c44	146-159	GAAR <b>A</b> L <b>H</b> G <b>V</b> R <b>V</b> L <b>E</b> A
c19	57-70 (1b)	Q <b>P</b> R <b>G</b> R <b>R</b> Q <b>P</b> I <b>P</b> K <b>A</b> R <b>Q</b> A	c45	151-164	LA <b>H</b> G <b>V</b> R <b>V</b> L <b>E</b> D <b>G</b> V <b>N</b> Y <b>A</b>
c20	62-75 (1a)	R <b>Q</b> P <b>I</b> P <b>K</b> V <b>R</b> R <b>E</b> G <b>R</b> T <b>A</b>	c46	155-168	VR <b>V</b> L <b>E</b> D <b>G</b> V <b>N</b> Y <b>A</b> T <b>G</b> N <b>A</b>
c21	62-75 (1b)	R <b>Q</b> P <b>I</b> P <b>K</b> A <b>R</b> Q <b>E</b> G <b>R</b> A	c47	160-173	D <b>G</b> V <b>N</b> Y <b>A</b> T <b>G</b> N <b>L</b> P <b>G</b> C <b>S</b> A
c22	67-80 (1a)	K <b>V</b> R <b>R</b> PE <b>G</b> R <b>T</b> W <b>A</b> Q <b>P</b> G <b>A</b>	c48	165-178	A <b>T</b> G <b>N</b> L <b>P</b> G <b>C</b> S <b>F</b> S <b>I</b> F <b>L</b> A
c23	67-80 (1b)	K <b>A</b> R <b>Q</b> PE <b>G</b> R <b>A</b> W <b>A</b> Q <b>P</b> G <b>A</b>	c49	169-182	L <b>P</b> G <b>C</b> S <b>F</b> S <b>I</b> F <b>L</b> L <b>A</b> L <b>L</b> A
c24	71-84 (1a)	PE <b>G</b> R <b>T</b> W <b>A</b> Q <b>P</b> G <b>Y</b> P <b>W</b> P <b>A</b>	c50	173-186	S <b>F</b> S <b>I</b> F <b>L</b> L <b>A</b> L <b>L</b> S <b>C</b> L <b>T</b> A
c25	71-84 (1b)	PE <b>G</b> R <b>A</b> W <b>A</b> Q <b>P</b> G <b>Y</b> P <b>W</b> P <b>A</b>	c51	178-191 (1a)	L <b>L</b> L <b>L</b> S <b>C</b> L <b>T</b> V <b>P</b> A <b>S</b> A
c26	76-89	W <b>A</b> Q <b>P</b> G <b>Y</b> P <b>W</b> P <b>L</b> Y <b>G</b> N <b>E</b> A	c52	178-191 (1b)	L <b>L</b> L <b>L</b> S <b>C</b> L <b>T</b> I <b>P</b> A <b>S</b> A

\*The underlined Alanine was added to peptides for synthesis convenience. The amino acids differing in sequences 1a and 1b are indicated in bold in genotype 1b peptides.

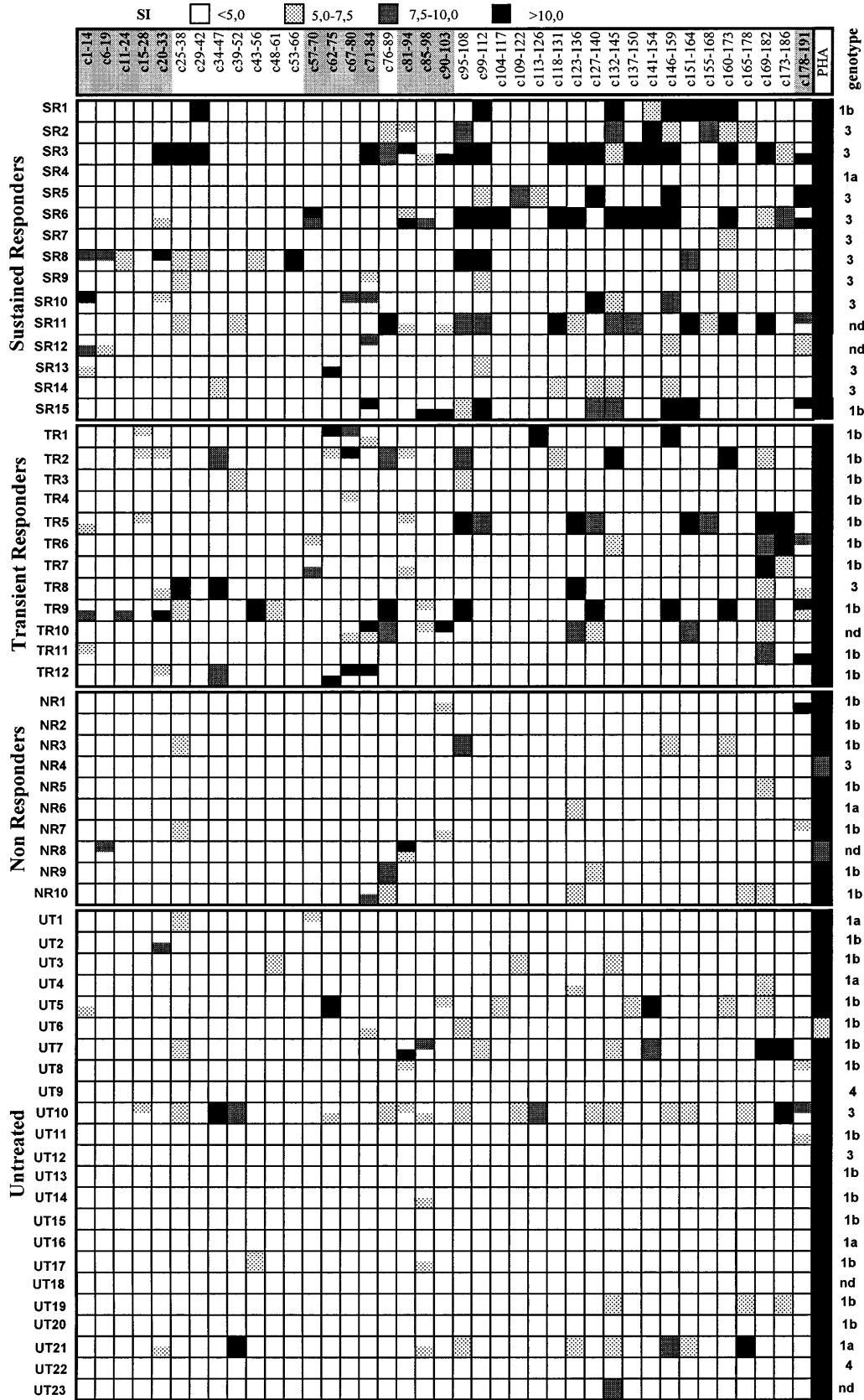


FIG. 3. IL-2 production in response to HCV core peptides by PBMC from patients with chronic hepatitis C and controls. Rows correspond to patients, and columns correspond to peptides. Results are presented as the SI (mean cpm with peptide/mean cpm without peptide). Higher SI are indicated with darker shades of gray. The recognition of peptides from conserved areas is indicated by a full square, while recognition of peptides from variable regions (peptides in bold) is indicated in two halves: the upper part of each square corresponds to sequence 1a, and the lower part corresponds to sequence 1b. The response of each patient to PHA is also indicated. HCV genotypes are specified on the right side of the figure.

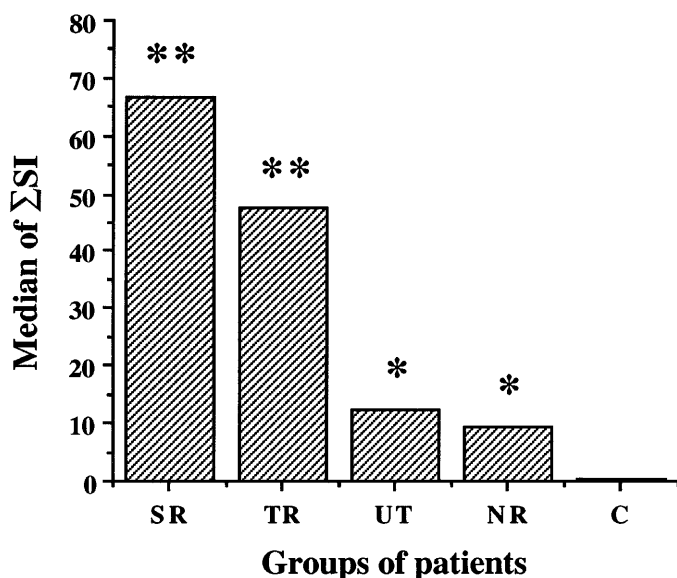


FIG. 4. Median value of  $\Sigma$ SI obtained with peptides eliciting positive Th response ( $SI \geq 5$ ) in patients from each group. C, healthy controls. \*\* $P < .001$  vs. C;  $P < .01$  vs. NR;  $P < .05$  vs. UT; \* $P < .05$  vs. C.

viremia after completion of IFN- $\alpha$  treatment (9 of 15 SR vs. 1 of 22 NR + TR responded to c99-112, and 9 of 15 SR vs. 3 of 22 NR + TR responded to c146-159). Using a logistic regression model, peptides c99-112 and c146-159 were found to be independently associated with sustained response to interferon ( $P < .005$  and  $P < .01$ , respectively), with adjusted odds ratios of 42.1 (95% CI: 3.4-526.5) and 13.2 (95% CI: 1.7-100.5), respectively. Response to at least one of these two peptides was observed in 12 of 15 (80%) patients with sustained response, but only in 4 of 22 (18.1%) patients who remained viremic after completing IFN- $\alpha$  treatment (TR + NR) ( $P = .0001$ ; combined odds ratio: 31.7; 95% CI: 4.5-221.7). Analysis of HLA-DR molecules did not reveal any association between the response to these peptides and the presence of specific HLA-DR molecules in the groups of patients studied (data not shown). Thus, peptides c99-112 and c146-159 induced positive IL-2 response in patients with different HLA-DR molecules, suggesting that these peptides are recognized in the context of different HLA-DR alleles.

We observed that PBMC from patients infected with viral genotypes other than 1a and 1b were able to produce detectable amounts of IL-2 when stimulated with core peptides from sequences 1a and 1b. This can be clearly seen in the SR group, in which most of the patients were infected with genotype 3a (Fig. 3).

**Viral Load and Core-Specific Th Precursors.** In cohort A, viremia was quantified in the same sample used for immunological studies. When all viremic patients were included, no relationship was found between Th precursor frequency and viral load (Fig. 5A). However, when only patients with low viremia (below  $1 \times 10^7$  copies per milliliter;  $n = 7$ ) were considered, a close inverse correlation emerged between precursor frequency and viral load ( $r = .89$ ;  $P = .007$ ) (Fig. 5B).

#### DISCUSSION

In chronic hepatitis C, the contribution of specific T-cell immunity to the resolution of HCV infection following IFN- $\alpha$

therapy has not yet been clarified. The present article is intended to provide information on this point.

Our results show that patients manifesting sustained biochemical and virological response to interferon (SR group) possess a number of core-specific Th precursors 34 times higher than those who fail to clear HCV (NR + TR). It was of interest that NR and TR, despite being in contact with the virus for many years, have a Th precursor frequency similar to that of healthy HCV-seronegative controls. The situation was different in untreated patients; these subjects present a wide range of core-specific Th precursor frequencies, with a median value that was significantly lower than that of SR, but significantly higher than NR + TR. Longitudinal studies are needed to evaluate the changes in Th precursor frequency

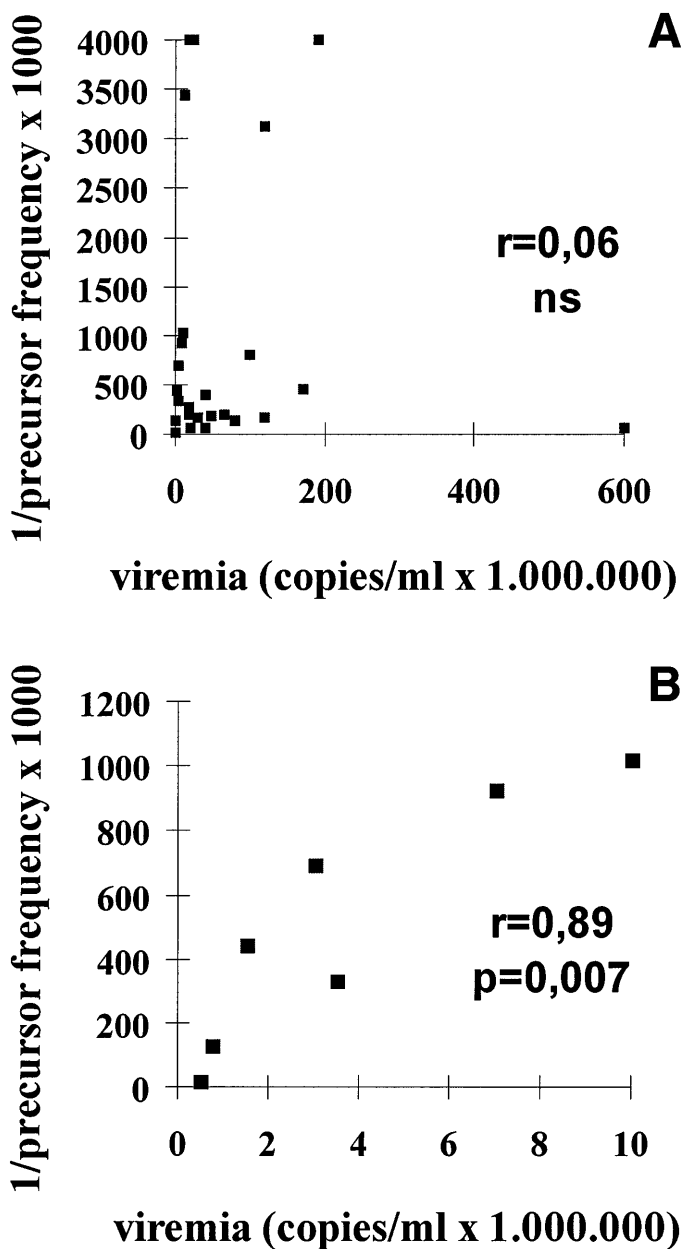


FIG. 5. Relationship between viral load and frequency of Th cell precursors against core in all viremic patients from cohort A (UT and NR after IFN therapy;  $n = 25$ ) (A) and in those patients with low viremia (below  $1 \times 10^7$  copies per milliliter;  $n = 7/25$ ) (B).

that occur during IFN- $\alpha$  therapy and the relationship between these changes and the ultimate treatment outcome.

In addition to these findings, we observed that the number of core peptides recognized by Th increased progressively in NR, UT, TR, and SR (from  $2.0 \pm 0.5$  in NR to  $8.2 \pm 1.5$  in SR), and that the intensity of the Th response against core peptides (as estimated by SI) was higher in SR than in UT or NR. Thus, our data indicate that sustained response to IFN- $\alpha$  is associated with both an expanded number of Th cell precursors against HCV core antigen and an extensive recognition of HCV core epitopes. In contrast, failure to clear the virus is associated with a low precursor frequency, comparable with that found in subjects without previous contact with the virus.

Our results are in accordance with reports from other authors,<sup>16,29-31</sup> indicating that Th responses to different viral antigens (including core) were associated with a benign course of HCV infection. Along the same lines, Sarobe et al.<sup>23</sup> found that the response to synthetic peptides from HCV-E1 protein was higher (in terms of number of peptides recognized and value of the SI) in those patients who resolved the infection after IFN- $\alpha$  therapy than in those who failed to clear the virus. Hoffman et al.<sup>32</sup> also observed that CD4<sup>+</sup> lymphocyte response against HCV proteins was more common in patients who responded to therapy than in nonresponders, and Iwata et al.<sup>33</sup> demonstrated a greater production of IFN- $\gamma$  in response to core in patients who eliminated HCV infection after IFN- $\alpha$  treatment, further emphasizing the role of Th cells in IFN-induced HCV clearance.

The wide range of determinants recognized by T cells in SR patients (Fig. 3) may be important in the prevention of the appearance of escape mutants, thus facilitating the elimination of the infection. It is of interest to note that SR patients recognized peptides from genotypes 1a and 1b, despite the fact that many of them were infected with genotype 3. This finding indicates a high degree of cross-reactivity between peptides of different genotypes in this highly conserved viral protein.<sup>34</sup> Similar results, showing cross-reactivities between peptides from different genotypes have been reported by others.<sup>16,35</sup>

In addition to quantitative aspects, the specificity of the Th response may also play a role in the elimination of the infection. Recently, Diepolder et al.<sup>36</sup> identified a peptide from NS3 protein (amino acids 1248 to 1261), associated with viral clearance in self-limited acute infection, that can be presented to CD4<sup>+</sup> cells by a great number of HLA-DR alleles. Similarly, Lechmann et al.,<sup>37</sup> using HCV-core 25-mer peptides, observed an association between the proliferative response to peptide CT4 (amino acids 148-172) and viral clearance. Using shorter synthetic peptides, we found that peptides c146-159 (encompassed by CT4) and c99-112, both corresponding to highly conserved regions,<sup>34</sup> showed an association with resolution of the infection after IFN- $\alpha$  therapy. Although the number of patients is not high enough to draw solid conclusions, the peptides described here may deserve consideration in the design of future T-cell-targeted immunotherapies for HCV infection.

As mentioned, vigorous Th anti-core reactivity was found in SR even years after completion of the therapy. This finding is in agreement with results from Ferrari et al.,<sup>38</sup> who showed that asymptomatic anti-HCV-positive subjects who successfully cleared HCV RNA after a previous episode of acute HCV infection express strong T-cell responses to HCV antigens

years after resolution of hepatitis. In hepatitis B virus infection, it has been postulated that sterilizing immunity frequently fails to occur after recovery from acute hepatitis, and that traces of virus can maintain an activated immune response for decades following clinical recovery, keeping the virus under control.<sup>39</sup> This could also be the situation in HCV infection after viral clearance following IFN- $\alpha$  therapy.

Finally, it should be mentioned that no clear relationship exists between viral load and the frequency of Th precursors when all viremic patients are considered. However, a close inverse relationship was found when only patients with viremia below  $1 \times 10^7$  copies per milliliter were considered. Although the analysis in this subgroup of 7 patients might be artificial, one could speculate that Th response is effective only in controlling low levels of viral replication. A similar result has been found by Rehmann et al.<sup>35</sup> and by Hiroishi et al.<sup>40</sup> when they studied cytotoxic T lymphocyte responses in patients with chronic HCV infection. In this context, IFN- $\alpha$  might act simultaneously by reducing viremia and by stimulating a T-cell immune response that might be effective only in the setting of low viral replication.

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