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 ± 1.5, 6.5 ± 1.2, 2.0 ± 0.5, 2.7 ± 0.9, and 0.3 ± 0.2, respectively. In SR, the intensity of the proliferative response to core peptides as estimated by the summation of stimulation indexes (ΣSI) 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.[1-3] 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.[4,5] Interferon alfa (IFN-α) 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.[6] Thus, only around 15% to 20% of cases experience sustained biochemical and virological response to treatment.[7] IFN-α has both direct antiviral activity and immunomodulatory properties,[8] 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.[9] 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.[9,10] 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-α 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,[11-13] 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.[14] Because T-cell help seems to play a role in the spontaneous resolution of HCV infection,[15,16] we hypothesized that Th responses might be important for viral clearance following IFN-α 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-α.

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-α 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-α 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-α therapy (sustained response [SR]); patients who failed to normalize serum transaminases during treatment with IFN-α 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-α therapy, 11 patients who failed to exibit 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-α therapy (SR) and those who remained viremic after completion of the treatment (NR + TR). Patients were studied 36.7 ± 16.9 and 27.8 ± 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 ± 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 ± 3.7 years) was also studied as a control group (C). Patients were investigated 7.2 ± 5.4, 9.4 ± 6.9, and 8.3 ± 5.8 months after completion of IFN-α 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,[17] 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[18] using the Fmoc alternative,[19] utilizing a manual multiple solid-phase peptide synthesizer.[20] The ninhydrin test of Kaiser was used to monitor every step.[21] 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 (2×10^5, 1×10^5, 5×10^4, and 2.5×10^4) were placed in 20 replica cultures (for each dilution) in RPMI-1640 medium containing L-glutamine (2 mmol/L), gentamicin (10 μg/mL), penicillin (50 U/mL), streptomycin (50 μg/mL), and HEPES (5 mmol/L), supplemented with 10% heat-inactivated human AB serum, in the presence of 1 μg/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 2×10^5 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.[22,23] Mitogen stimulation of cells (positive control) was performed by incubation with phytohaemaglutinin (PHA) (1/200 final dilution; Gibco BRL, Grand Island, NY). After 7 days of culture at 37°C and 5% CO_2, 50 μL 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 (5×10^{-5} mol/L), and were plated (5×10^3 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 μCi per well of [^{3}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

	Cohort A				Cohort B			
Response to IFN	UT	NR	TR	SR	UT	NR	TR	SR
Number of patients	20	5	6	12	23	10	12	15
Age (yr)	35.3 ± 11	50.8 ± 19.5	50.8 ± 14.5	44.7 ± 14	53.8 ± 5.1	47.5 ± 3.9	40.3 ± 4	39.5 ± 11.1
Sex (male/female)	14/6	2/3	2/4	9/3	9/14	5/5	7/5	12/3
Viral load (×10^6)*	82.5 ± 156	59.3 ± 93.9	36.8 ± 39.5	12.2 ± 2.8	78.6 ± 162.5	170.4 ± 198.8	30.7 ± 31.4	13.9 ± 26.7
ALT	62.8 ± 46.4	124 ± 137.1	113.2 ± 65.9	15.1 ± 4.8	92.5 ± 85.1	74.9 ± 28.5	86.7 ± 93.4	13.8 ± 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-α treatment.

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

‡Patient coinfected 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[24] (evaluation according to χ^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 2×10^5 cells per well, and cultured in triplicate in 96-well flat-bottomed plates with 10 μg/mL of an anti–IL-2 receptor monoclonal antibody (BT563, Biotest Pharma) in the presence or absence of synthetic peptides at 25 μg/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°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 SI ≥ 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.[25,26] Genotyping was performed using a hybridization technique with specific probes for HCV genotypes 1a, 1b, 2a, 2b, and 3a according to Simmonds et al.,[27] with the amplified HCV core region by nested PCR as described by Viazov et al.[28] with the following technical modifications: primers for the second PCR were 5′-digoxigenin–labeled, and hybridization was detected using an anti–digoxigenine peroxidase–labeled antibody (Boehringer-Mannheim, Mannheim, Germany). HCV RNA was quantified by a competitive PCR technique as previously reported.[25,26]

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 ± 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-α 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 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 patients, except one, had precursor frequencies above 1 in 1.5×10^5 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 HVC-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.

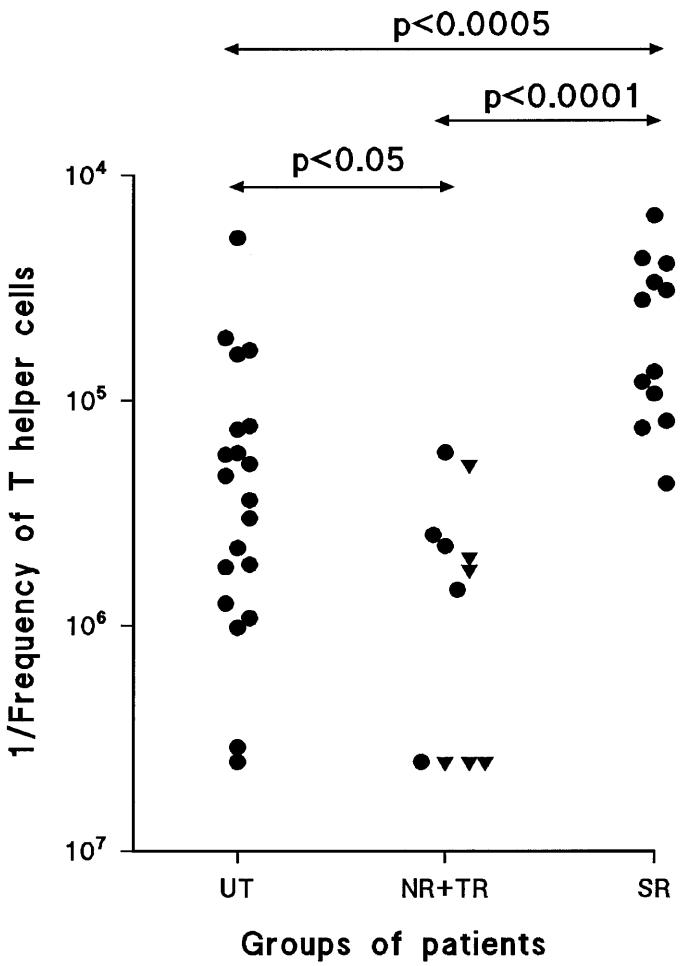

FIG. 1. Frequency of Th cell precursors (IL-2–producing cells) recognizing HCV core protein in the PBMC population from different groups of patients with chronic hepatitis C classified according to the response to IFN-α treatment. Calculation of the frequency of Th precursors was performed by LDA. UT, 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-α withdrawal; SR, patients with sustained response to interferon. (▼), correspond to TR patients.

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

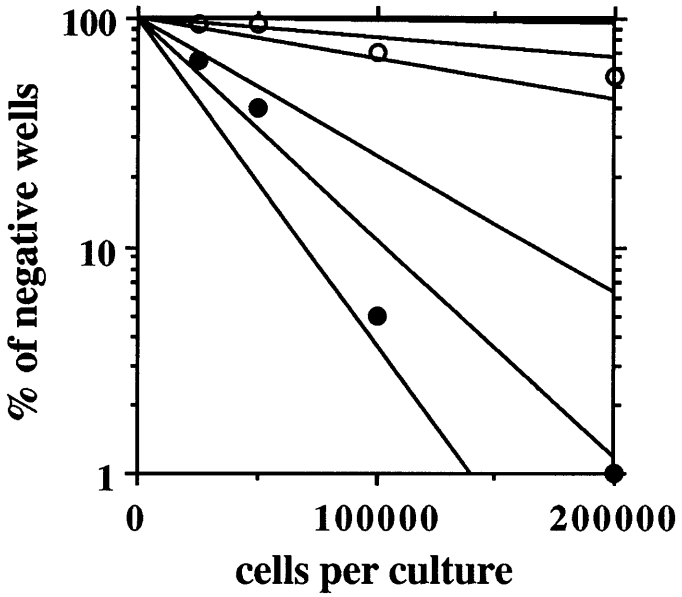

FIG. 2. Comparison of Th cell precursors in two representative cases of SR and NR patients using LDA. The results are shown as a semilogarithmic plot of responder cell input per well against the percentage of negative wells. 95% confidence intervals are represented by *dotted lines*. (●), SR; (○), NR.

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-α 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 ± 1.5, 6.5 ± 1.2, 2.7 ± 0.9, 2.0 ± 0.5, and 0.3 ± 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 ΣSI of peptides eliciting a positive response (SI ≥ 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)	MSTIPKPQRKTKRNA	c27	81-94 (1a)	YPWPLYGNEGCGWA
c2	1-14 (1b)	MST**N**PK**EF**RKTKRNA	c28	81-94 (1b)	YPWPLYGNEG**M**GWA
c3	6-19 (1a)	KPQRKTKRNTNRRPA	c29	85-98 (1a)	LYGNEGCGWAGWLLA
c4	6-19 (1b)	K**EF**RKTKRNT**L**RRPA	c30	85-98 (1b)	LYGNEG**M**GWAGWLLA
c5	11-24 (1a)	TKRNTNRRPQDVKFA	c31	90-103 (1a)	GCGWAGWLLSPRGSA
c6	11-24 (1b)	TKRNT**L**RRPQDVRFA	c32	90-103 (1b)	G**M**GWAGWLLSPRGSA
c7	15-28 (1a)	TNRRPQDVKFPGGGA	c33	95-108	GWLLSPRGSRPSWGA
c8	15-28 (1b)	T **L**RRPQDV**R**FPGGGA	c34	99-112	SPRGSRPSWGPTDPA
c9	20-33 (1a)	QDVKFPGGGQIVGGA	c35	104-117	RPSWGPTDPRRRSRA
c10	20-33 (1b)	QDV**R**FPGGGQIVGGA	c36	109-122	PTDPRRRSRNLGKVA
c11	25-38	PGGGQIVGGVYLLPA	c37	113-126	RRRSRNLGKVIDTLA
c12	29-42	QIVGGVYLLPRRGPA	c38	118-131	NLGKVIDTLTCGFA
c13	34-47	VYLLPRRGPRLGVRA	c39	123-136	IDTLTCGFADLMGYA
c14	39-52	RRGPRLGVRATRKTA	c40	127-140	TCGFADLMGYIPLVA
c15	43-56	RLGVRATRKTSERSA	c41	132-145	DLMGYIPLVGAPLGA
c16	48-61	ATRKTSERSQPRGRA	c42	137-150	IPLVGAPLGGAARA
c17	53-66	SERSQPRGRRQPIPA	c43	141-154	GAPLGGAARALAHGA
c18	57-70 (1a)	QPRGRRQPIPKVRRA	c44	146-159	GAARALAHGVRVLEA
c19	57-70 (1b)	QPRGRRQPIPK**A**RQA	c45	151-164	LAHGVRVLEDGVNYA
c20	62-75 (1a)	RQPIPKVRRPEGRTA	c46	155-168	VRVLEDGVNYATGNA
c21	62-75 (1b)	RQPIPK**AR**Q**R**PEGR**A**	c47	160-173	DGVNYATGNLPGCSA
c22	67-80 (1a)	KVRRPEGRTWAQPGA	c48	165-178	ATGNLPGCSFSIFLA
c23	67-80 (1b)	K**AR**Q**R**PEGR**A**WAQPGA	c49	169-182	LPGCSFSIFLLALLA
c24	71-84 (1a)	PEGRTWAQPGYPWPA	c50	173-186	SFSIFLLALLSCLTA
c25	71-84 (1b)	PEGR**A**WAQPGYPWPA	c51	178-191 (1a)	LLALLSCLTVPASA
c26	76-89	WAQPGYPWPLYGNEA	c52	178-191 (1b)	LLALLSCLTIPASA

*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.

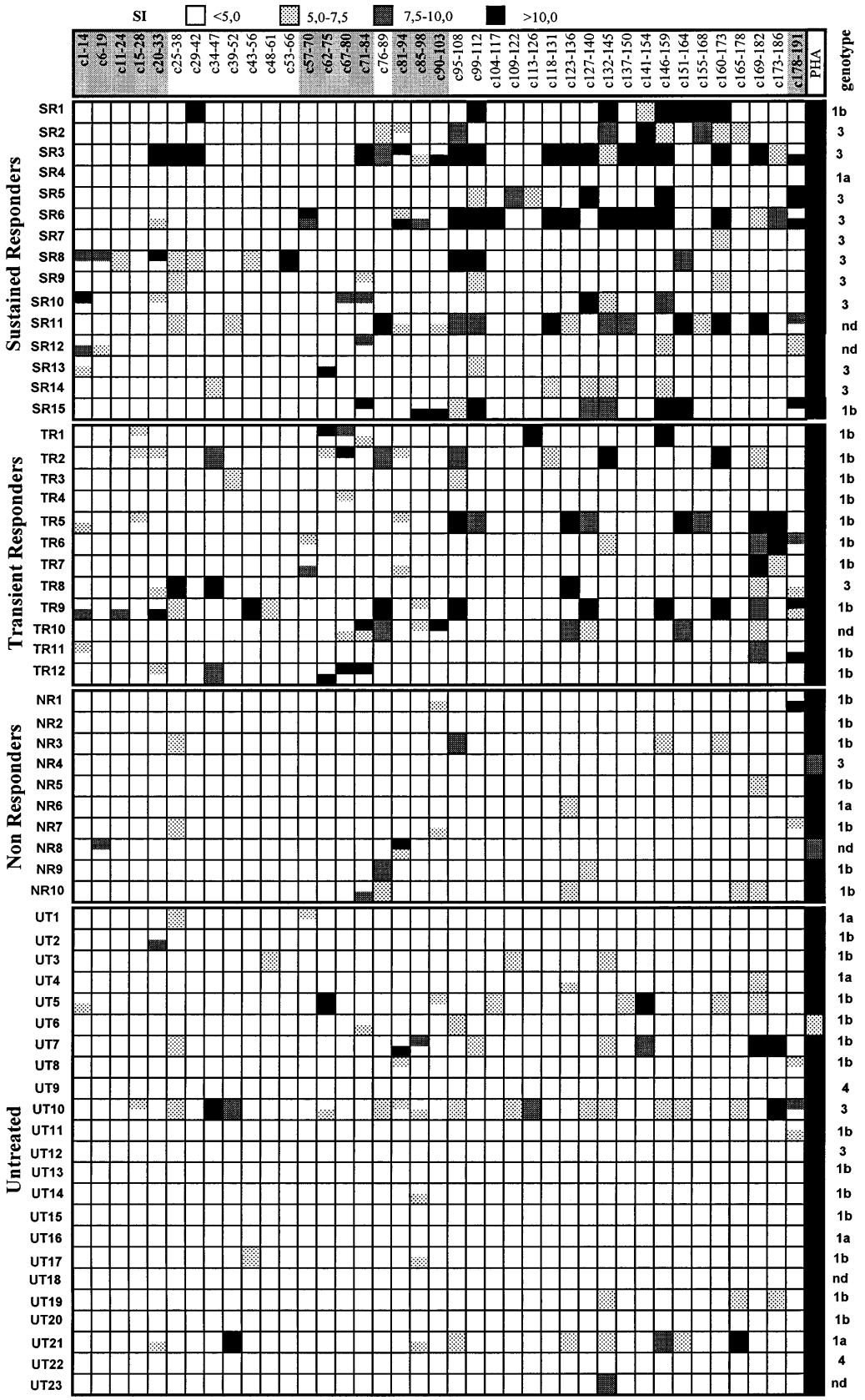

FIG. 3. IL-2 production in response to HCV core peptides by PBMC from patients with chronic hepatitis C and controls. *Rows* correspond to patients, and *columns* correspond to peptides. Results are presented as the SI (mean cpm with peptide/mean cpm without peptide). Higher SI are indicated with *darker 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.

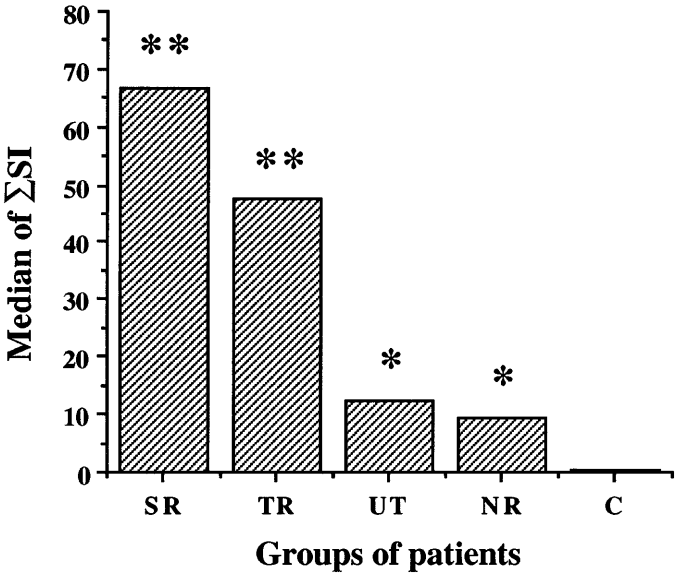

FIG. 4. Median value of ΣSI obtained with peptides eliciting positive Th response (SI ≥ 5) in patients from each group. C, healthy controls. $^{**}P < .001$ vs. C; $P < .01$ vs. NR; $P < .05$ vs. UT; $^{*}P < .05$ vs. C.

viremia after completion of IFN-α 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-α 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 1×10^7 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-α 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

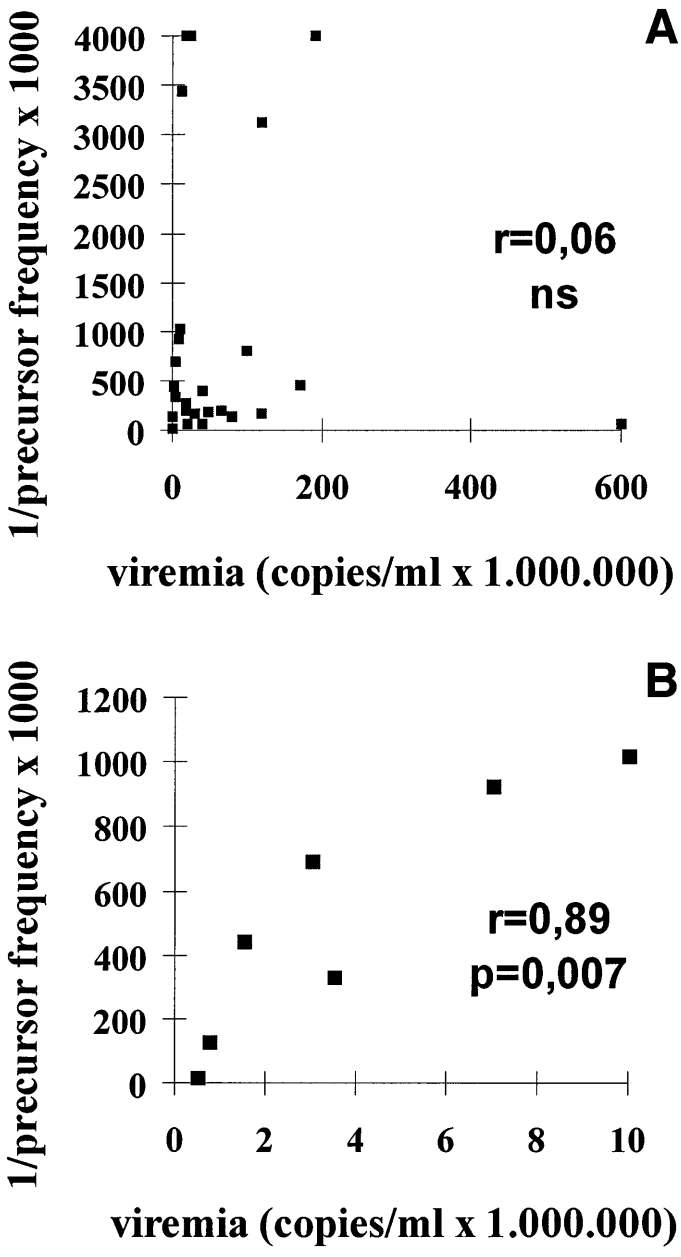

FIG. 5. Relationship between viral load and frequency of Th cell precursors against core in all viremic patients from cohort A (UT and NR after IFN therapy; n = 25) (A) and in those patients with low viremia (below 1×10^7 copies per milliliter; n = 7/25) (B).

that occur during IFN-α 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 ± 0.5 in NR to 8.2 ± 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-α 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,[16,29-31] 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.[23] 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-α therapy than in those who failed to clear the virus. Hoffman et al.[32] also observed that $CD4^+$ lymphocyte response against HCV proteins was more common in patients who responded to therapy than in nonresponders, and Iwata et al. [33] demonstrated a greater production of IFN-γ in response to core in patients who eliminated HCV infection after IFN-α 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.[34] Similar results, showing cross-reactivities between peptides from different genotypes have been reported by others.[16, 35]

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.[36] 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 $CD4^+$ cells by a great number of HLA-DR alleles. Similarly, Lechmann et al.,[37] 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,[34] showed an association with resolution of the infection after IFN-α 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.,[38] 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.[39] This could also be the situation in HCV infection after viral clearance following IFN-α 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 1×10^7 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 Rehermann et al.[35] and by Hiroishi et al.[40] when they studied cytotoxic T lymphocyte responses in patients with chronic HCV infection. In this context, IFN-α 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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REFERENCES

1. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989;244:359-362.
2. Houghton MH, Weiner AJ, Han J, Kuo G, Choo QL. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. HEPATOLOGY 1990;14:381-388.
3. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, et al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 1989;244:362-364.
4. Genesca J, Esteban JI, Alter HJ. Blood-borne non-A, non-B hepatitis: hepatitis C. Semin Liver Dis 1991;11:147-164.
5. Ruiz J, Sangro B, Cuende JI, Beloqui O, Riezu-Boj JI, Herrero JI, Prieto J. Hepatitis B and C viral infections in patients with hepatocellular carcinoma. HEPATOLOGY 1992;16:637-641.
6. Di Bisceglie AM, Hoofnagle JH. Therapy in chronic hepatitis C with alfa-interferon: the answer? Or more questions? HEPATOLOGY 1991;13:601-603.
7. Camps J, Castilla A, Ruiz J, Civeira MP, Prieto J. Randomised trial of limphoblastoid alpha-IFN in chronic hepatitis C. J Hepatol 1993;17:390-396.
8. Parronchi P, Mohapatra S, Sampognaro S, Giannarini L, Wahn U, Chong P, Maggi E, et al. Effects of interferon-alpha on cytokin profile, T cell receptor repertoire and peptide reactivity of human allergen-specific T cells. Eur J Immunol 1996;26:697-703.
9. Bukh J, Miller RH, Purcell RH. Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Semin Liver Dis 1995;15:41-63.
10. Weiner AJ, Geysen HM, Christopherson C, Hall JE, Mason TS, Saracco G, Bonino F, et al. Evidence for immune selection of hepatitis C virus (HCV) putative envelope glicoprotein variants: potential role in chronic HCV infections. Proc Natl Acad Sci U S A 1992;89:3468-3472.
11. Keene JA, Forman J. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. J Exp Med 1982;155:768-782.
12. Leist TP, Cobbold SP, Waldmann H, Aguet M, Zinkernagel RM. Functional analysis of T lymphocyte subsets. J Immunol 1987;138:2278-2281.

13. Leist TP, Koshler M, Zinkernagel M. Impaired generation of antiviral cytotoxicity against lymphocytic choriomeningitis and vaccinia virus in mice treated with CD4-specific monoclonal antibody. Scand J Immunol 1989;30:679-684.
14. Guidotti LG, Guilhot S, Chisari FV. Interleukin-2 and alpha/beta interferon down-regulate hepatitis B virus gene expression in vivo by tumor necrosis factor–dependent and –independent pathways. J Virol 1994;68:1265-1270.
15. Woitas RP, Lechmann M, Jung G, Kaiser R, Sauerbruch T, Spengler U. CD30 induction and cytokine profiles in hepatitis C virus core-specific peripheral blood T lymphocytes. J Immunol 1997;159:1012-1018.
16. Missale G, Bertoni R, Lamonaca V, Valli A, Massari M, Mori C, Rumi MG, et al. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. J Clin Invest 1996;98:706-714.
17. Martinot Peignoux M, Marcellin P, Pouteau M, Castelnau C, Boyer N, Poliquin M, Degott C, et al. Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alfa therapy in chronic hepatitis C. HEPATOLOGY 1995;22:1050-1056.
18. Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc 1963;18:80-84.
19. Atherton E, Logan JC, Sheppard RC. Peptide synthesis. Part 2. Procedures for solid phase synthesis using N-fluorenil metoxicarbonil amino-acids on polyamide supports. Synthesis of substance P and of acyl carrier protein 65-74 decapeptide. J Chem Soc Perkin Trans 1981;1:538-546.
20. Borrás-Cuesta F, Golvano J, Sarobe P, Lasarte JJ, Prieto I, Szabo A, Guillaume JL, et al. Insights on the aminoacid side-chain interactions of a T-cell determinant. Biologicals 1991;19:187-190.
21. Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid phase synthesis of peptides. Anal Biochem 1970;34:595-598.
22. Clerici M, Stocks NI, Zajac RA, Boswell RN, Bernstein DC, Mann DL, Shearer GM, et al. Interleukin-2 production used to detect antigenic peptide recognition by T-helper lymphocytes from asymptomatic HIV-seropositive individuals. Nature 1989;339:383-385.
23. Sarobe P, Jauregui JI, Lasarte JJ, García N, Civeira MP, Borrás-Cuesta F, Prieto J. Production of interleukin-2 in response to synthetic peptides from hepatitis C virus E1 protein in patients with chronic hepatitis C: relationship with the response to interferon treatment. J Hepatol 1996;25:1-9.
24. Waldmann H, Penhale WJ, Sedgwick JD. Limiting dilution analysis. In: Klaus GGB, ed. Lymphocytes: A Practical Approach. Oxford: IRL Press, 1987:163-188.
25. Larrea E, García N, Quian C, Civeira MP, Prieto J. Tumor necrosis factor a gene expression and the response to interferon in chronic hepatitis C. HEPATOLOGY 1996;23:210-217
26. Gavier B, Martínez-Gonzalez MA, Riezu-Boj JI, Lasarte JJ, García N, Civeira MP, Prieto J. Viremia after one month of interferon therapy predicts treatment outcome in chronic hepatitis C. Gastroenterology 1997;113:1647-1653.
27. Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, Brouwer JT, et al. A proposed system for the nomenclature of hepatitis C viral genotypes. HEPATOLOGY 1994;19:1321-1324.
28. Viazov S, Zibert A, Ramakrishnan K, Widell A, Cavicchini A, Schreier E, Roggendorf M. Typing of hepatitis C virus isolates by DNA enzyme immunoassay. J Virol Methods 1994;48:81-92.
29. Botarelli P, Brunetto MR, Minutello MA, Calvo P, Unutmaz D, Weiner AJ, Choo QL, et al. T lymphocyte response to hepatitis C virus in different clinical courses of infection. Gastroenterology 1993;104:580-587.
30. Ferrari C, Valli A, Galati L, Penna A, Scaccaglia P, Giuberti T, Schianchi C, et al. T-cell response to structural and nonstructural hepatitis C virus antigens in persistent and self-limited hepatitis C virus infections. HEPATOLOGY 1994;19:286-295.
31. Diepolder HM, Zachoval R, Hoffmann RM, Wierenga RA, Santantonio T, Jung MC, Eicherlaub D, et al. Possible mechanism involving T lymphocyte response to non-structural preotein NS3 in viral clearance in acute hepatitis C infection. Lancet 1995;346:1006-1007.
32. Hoffmann-RM, Diepolder-HM, Zachoval-R, Zwiebel-FM, Jung-MC, Scholz-S, Nitschko-H, et al. Mapping of immunodominant $CD4^+$ T lymphocyte epitopes of hepatitis C virus antigens and their relevance during the course of chronic infection. HEPATOLOGY 1995;21:632-638.
33. Iwata K, Wakita T, Okumura A, Yoshioka K, Takayanagi M, Wands JR, Kakumu S. Interferon gamma production by peripheral blood lymphocytes to hepatitis C virus core protein in chronic hepatitis C infection. HEPATOLOGY 1995;22:1057-1064.
34. Bukh J, Purcell RH, Miller RH. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc Natl Acad Sci U S A 1994;91:8239-8243.
35. Rehermann B, Chang KM, McHutchinson J, Kokka R, Houghton M, Rice CM, Chisari FV. Differential cytotoxic T-lymphocyte responsiveness to the hepatitis B and C viruses in chronically infected patients. J Virol 1996;70:7092-7102.
36. Diepolder HM, Gerlach JT, Zachoval R, Hoffmann RM, Jung MC, Wierenga EA, Scholz S, et al. Immunodominant $CD4^+$ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. J Virol 1997;71:6011-6019.
37. Lechmann M, Ihlenfeldt HG, Braunschweiger I, Giers G, Jung G, Matz B, Kaiser R, et al. T-and B-cell responses to different hepatitis C virus antigens in patients with chronic hepatitis C infection and in healthy anti-hepatitis C virus-positive blood donors without viremia. HEPATOLOGY 1996;24:790-795.
38. Ferrari C, Valli A, Galati L, Penna A, Scaccaglia A, Giuberti T, Schianchi C, et al. T-cell response to structural and nonstructural hepatitis C virus antigens in persistent and self-limited hepatitis C virus infections. HEPATOLOGY 1994;19:286-295.
39. Rehermann-B, Ferrari-C, Pasquinelli-C, Chisari-FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. Nat Med 1996;2:1104-1108.
40. Hiroishi K, Kita H, Kojima M, Okamoto H, Moriyama T, Kaneko T, Ishikawa T, et al. Cytotoxic T lymphocyte response and viral load in hepatitis C virus infection. HEPATOLOGY 1997;25:705-712.