Analysis of hepatitis C virus-specific CD8+ T-cells with HLA-A*24 tetramers during phlebotomy and interferon therapy for chronic hepatitis C

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Abstract. Hepatitis C virus (HCV)-specific, HLA class I-restricted, CD8-positive (CD8+) T lymphocytes are thought to contribute to viral clearance as well as liver disease in chronic hepatitis C. For the patients who do not respond to interferon (IFN) therapy, phlebotomy can be used as a tool to reduce inflammation and lower transaminase levels; however, the immunological aspects have not been clearly defined. In this study, we evaluated the HCV-specific CD8+ T-cell responses during phlebotomy and IFN therapy using HLA-A*24 tetramers in 6 Japanese patients with chronic hepatitis C. During phlebotomy, 4 of the 6 cases achieved a biochemical response, but there was no clear correlation between its efficacy and HCV viral loads or changes in frequencies or activation status of tetramer-positive T-cells. In contrast, the frequencies of tetramer-positive cells and the proportions of T-cells expressing activation marker HLA-DR were higher in sustained viral responders than in transient responders to IFN therapy. Furthermore, expression of the activation marker was enhanced in the initial period of IFN therapy. The results suggest that the immunological aspects of phlebotomy obviously differ from those of IFN therapy and these differences may provide clues as to a therapeutic strategy of their combination for patients who do not respond to IFN monotherapy.

Introduction

Interferon (IFN) treatment is a radical therapy for the elimination of hepatitis C virus (HCV), but many patients do not respond to it; so called 'non-responders'. There have been recent advances in treatment, such as combination therapy with Peginterferon α-2a or α-2b and ribavirin (1-4). To date,

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there are no therapies for HCV elimination with a sufficiently high success rate and low rate of adverse events.

For non-responders to IFN therapy, secondary treatment is needed to lower serum transaminase levels, slow the progression of fibrosis and reduce the occurrence of hepato-cellular carcinoma (5). Phlebotomy is one of the treatments used to reduce inflammation and lower serum transaminase levels (6).

Several studies have examined the correlation between HCV and iron levels. Smith *et al* reported that the progression of fibrosis is faster in patients with chronic hepatitis C with congenital hemochromatosis than in those with normal iron levels (7). Fontana *et al* reported that combination therapy with IFN and phlebotomy may be more effective than IFN monotherapy in patients with chronic hepatitis C (8). We reported the hemosiderin deposition may be a predictive parameter for the efficacy of IFN therapy (9). Mandishona *et al* reported that excess iron may promote the occurrence of hepatocellular carcinoma (10).

The mechanism by which phlebotomy decreases transaminase levels is thought to involve a decrease in the toxic effects of superoxide produced by iron excess (11). However, no studies have been reported regarding the immunological effects of phlebotomy that may be important in mitigating liver injury.

HCV-specific CD8+ cytotoxic T lymphocytes (CTLs) contribute to viral clearance in acute, self-limited hepatitis C as well as to liver cell injury in the more frequent cases with chronic hepatitis C (12-16). In a study using HLA-A*24 tetramer, we previously showed that a close correlation exists between the HCV-specific CD8+ T-cell profile and hepatic fibrosis in HCV-infected Japanese patients, most of whom are HLA-A*24 positive (17). In this study, we analyzed HCV-specific CD8+ T-cell responses during phlebotomy and IFN therapy and observed a correlation between changes in the HCV-specific CD8+ T-cell profile and the therapeutic effects of each treatment.

Materials and methods

Patients. Patients with chronic hepatitis C presented at Kanazawa University Hospital between June 2000 and June 2001 were included in this study. Their selection and diagnosis were based on the following criteria: 1) age from 20 to 70

Table I. Clinical characteristics of patients.

Patients	Age (years)	Sex (M/F)	HCV Serogroup	HCV-RNA (KIU/ml)	ALT (IU/l)	HAI Stage (F)	HAI Grade (A)	ALT change during phlebotomy	Response to IFN therapy
1	47	F	2	69	40	1	1	Not decreased	SVR
2	60	M	2	88	104	3	2	Decreased	TVR
3	43	M	1	>500	97	3	2	Decreased	TVR
4	65	M	1	1.9	80	1	1	Decreased	SVR
5	55	M	2	0.7	39	3	2	Decreased	SVR
6	51	M	2	>500	110	1	1	Not decreased	TVR

Serum HCV RNA was quantified with the Amplicore HCV Monitor ver.3. HAI, histological activitiy index; SVR, sustained viral responder; TVR, transient viral responder.
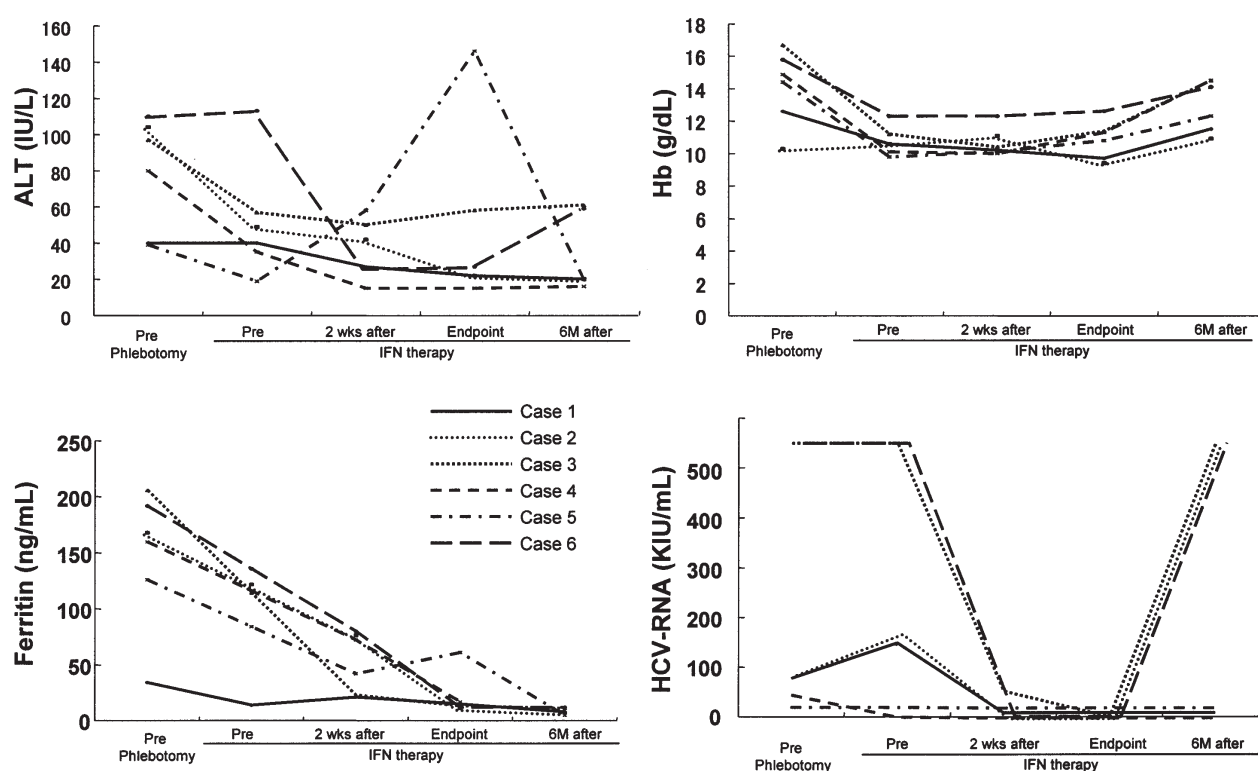

Figure 1. Trends over time for alanine aminotransferase (ALT), hemoglobin, ferritin and HCV-RNA levels during phlebotomy and interferon (IFN) therapy in patients with chronic hepatitis C. Phlebotomy was performed by removing 200 to 400 ml blood per session, once or twice weekly. The sessions were continued until achieving the target levels of hemoglobin (10 g/dl) and/or ferritin (10 ng/ml). After achieving the target levels, IFN-α-2b was administered at a dose of 6 to 10 MU per day for 2 weeks and then 3 times a week for 22 weeks. Each line indicates a single patient.

years; 2) elevated serum aminotransferase (ALT; >50 IU/l) at least once within 1 year; 3) hemoglobin greater than 13.0 g/dl in males or greater than 11.0 g/dl in females; 4) no liver cirrhosis; and 5) HLA-A*24 positive. All cases provided written informed consent.

Phlebotomy and IFN therapy. Phlebotomy was performed by removing 200 to 400 ml blood per session, once or twice weekly. The sessions were continued until achieving the target level of hemoglobin (10 g/dl) and/or ferritin (10 ng/ml). After achieving the target level, IFN-α-2b was administered at a dose of 6 to 10 MU per day for 2 weeks and then 3 times a week for 22 weeks.

Complete blood cell count, liver function tests, HCV-RNA determinations and T-cell analysis were performed pre-phlebotomy, just before IFN administration, 2 weeks after IFN

therapy, immediately after IFN therapy and 6 months after IFN administration.

Patients whose transaminases decreased during phlebotomy, were recorded as biochemical responders and the others as non-responders. With respect to the HCV-RNA level, patients whose HCV-RNA levels were undetectable both at the end-points of IFN therapy and even at 6 months after the IFN therapy completion were designated as sustained viral responders (SVR) and those whose HCV-RNA were undetectable at the end point of IFN therapy but reappeared 6 months after the end of IFN therapy were designated as transient viral responders (TVR) (1).

*Synthesis of HLA-A*2402-peptide tetramers.* Five peptides were selected to synthesize HLA-A*2402-peptide tetramers (17): HCV E2 717-725 (EYVLLLFLL), NS3 1292-1300
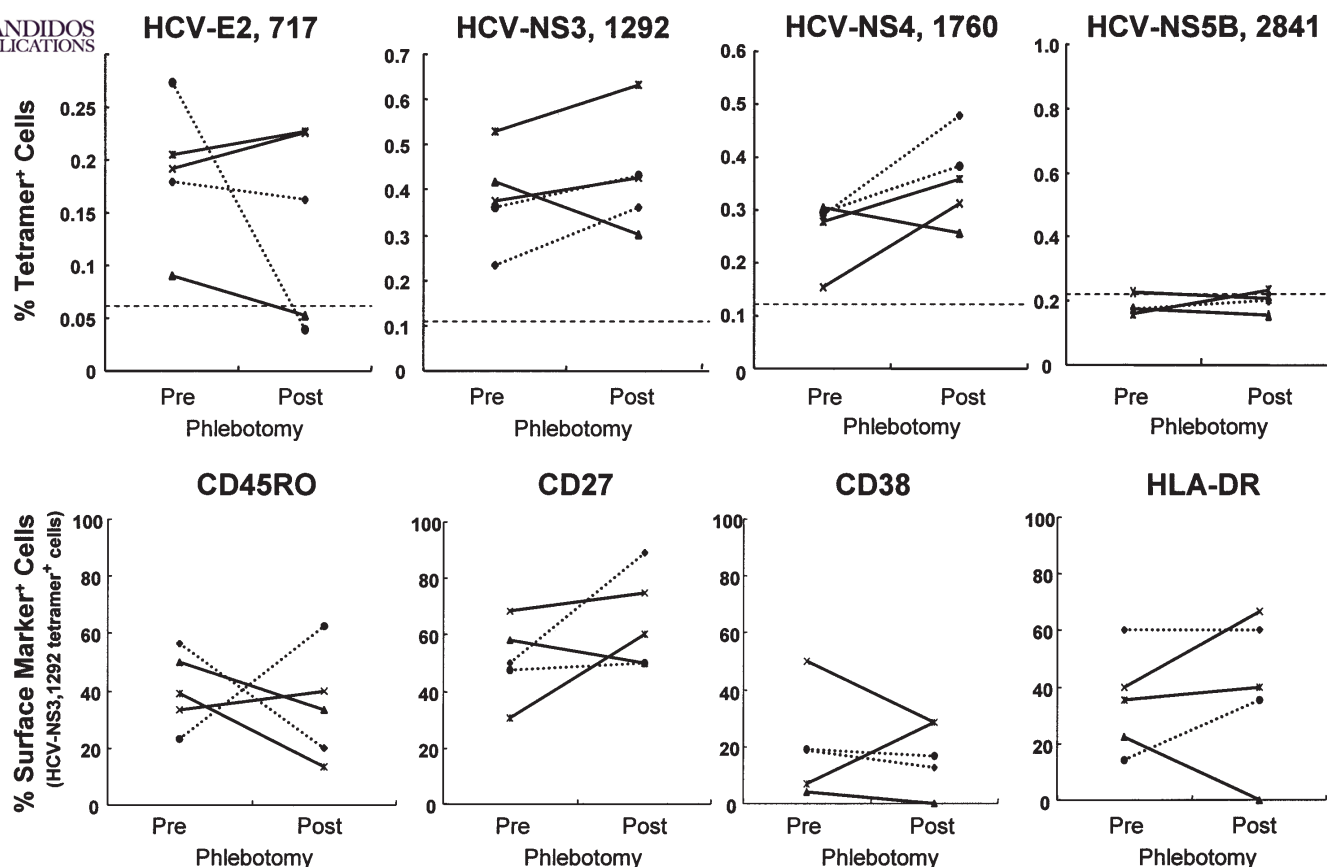
Figure 2. Changes in frequencies of four types of HLA-A*24 tetramer-positive cells and surface markers of tetramer HCV-NS3, 1292-positive cells during phlebotomy. Solid lines represent biochemical responders to phlebotomy and broken lines represent biochemical non-responders (subjects whose ALT did not decrease during phlebotomy). Horizontal broken lines indicate the cut-off value for each HLA tetramer, as mentioned in Materials and methods. There was no correlation between the biochemical effects of phlebotomy and the frequencies of tetramer-positive T-cells or the phenotypes of tetramer-positive cells.

(TYSTYGKFL), NS4 1760-1768 (FWAKHMWNF), NS5B 2841-2849 (RMILMTHFF) and NS5B 2870-2878 (CYSIEPLDL). Three of them, E2 717-725, NS3 1292-1300 and NS5B 2870-2878 were selected because they have been reported to bind to HLA-A*24 with good affinity (IC_{50} <500 nM) in a direct peptide binding assay (18). The other two peptides were chosen because they were conserved within the reported major HCV genotypes 1a and 1b sequences (19-21).

The cut-off values for positive staining with the tetramers was 2 SD above the mean for all control subjects studied previously (17): 0.064% for tetramer HCV-E2.717, 0.11% for tetramer HCV-NS3.1292, 0.12% for tetramer HCV-NS4 1760, 0.22% for tetramer HCV-NS5B.2841 and 0.10% for tetramer HCV-NS5B.2870.

Tetramer staining and flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by separation using Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. Freshly isolated PBMCs were stained with tetrameric complexes and antibodies and were then analyzed. The following monoclonal antibodies (mAbs) were used; anti-CD8-Cy-Chrome (CyC) (HIT8a), anti-CD-4-Allophycocyanin (APC) (SK3), anti-CD14-APC (MΦP9), anti-CD19-APC (SJ25C1), anti-CD45RA-FITC (HI100), anti-CD27-FITC (M-T271), anti-CD38-RITC (HIT2) and anti-HLA-DR-FITC (L243) (BD PharMingen, Sand Diego, CA). Freshly isolated

cells ($1x10^6$) were washed, resuspended in 200 μl PBS without calcium and phosphate, and stained with 40 μg/ml of tetrameric complexes for 30 min at room temperature. Subsequently, antibodies against cell surface proteins were added and incubated for an additional 30 min at room temperature. Cells were washed, fixed with 1% formalin/PBS, and analyzed on a FACSCalibur™ flow cytometer. Data were analyzed with CELLQuest™ software (Becton Dickinson, San Jose, CA).

Results

Clinical course. The 6 cases studied included 5 males and 1 female (Table I). They ranged in age from 43 to 60 years. Of the patients, 4 had serogroup 2 HCV and 2 had serogroup 1 HCV. Serogroup 1 HCV is known to be more common than serogroup 2 in Japan (22).

Phlebotomies were performed in all cases without significant adverse events over a period of 7 to 20 days. Eventually, the total volume of blood removed was from 600 to 2800 ml (mean = 1600 ml). In 4 of the 6 cases, transaminase levels decreased during phlebotomy, but there was no effect of phlebotomy on HCV viral loads (Fig. 1).

IFN treatments were associated with lower HCV viral loads. At the endpoints of the treatments, HCV-RNA disappeared from sera of all 6 cases. Six months after IFN therapy, HCV reappeared in 3 cases (transient viral responder,
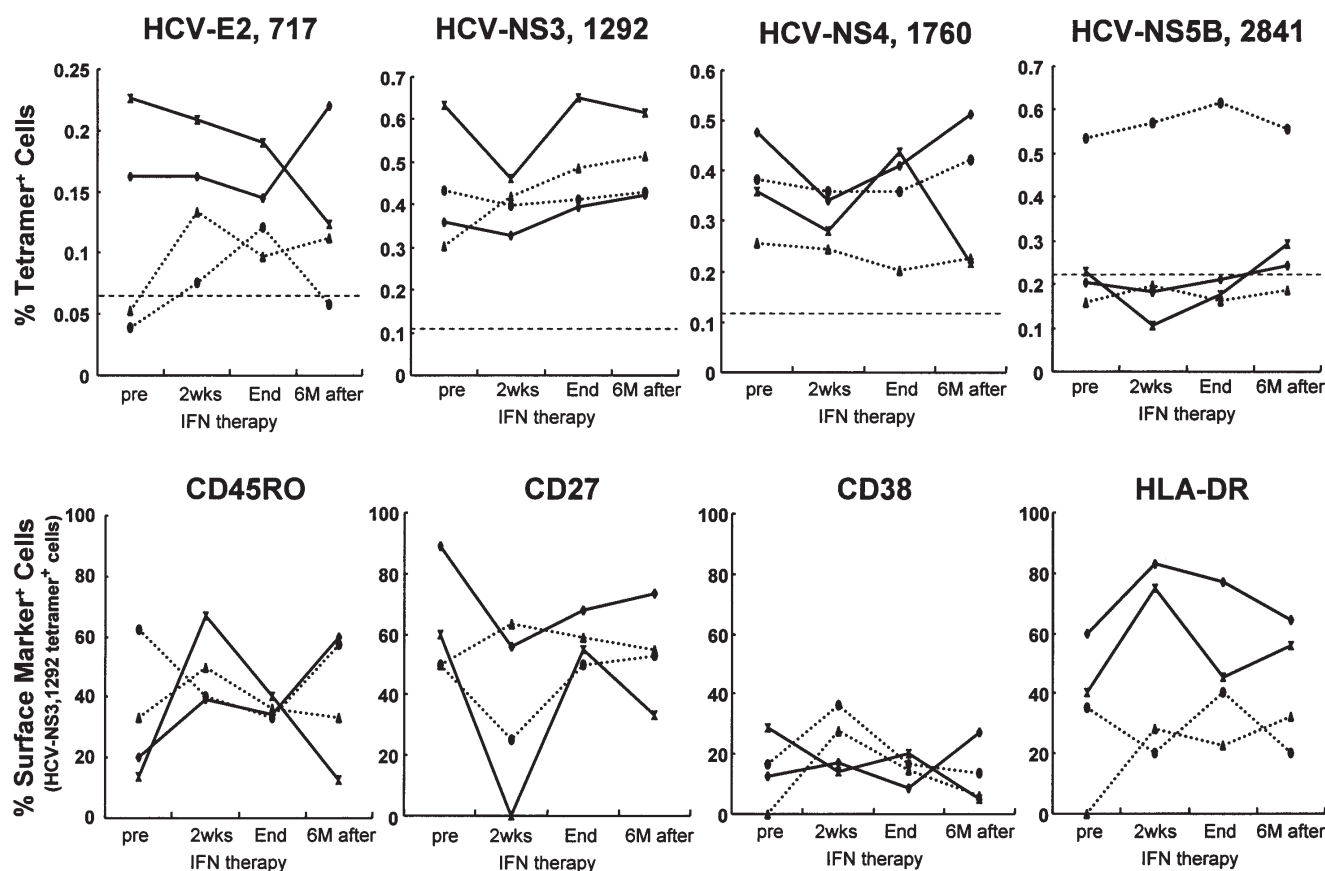
Figure 3. Changes in frequencies of four types of HLA-A*24 tetramer-positive cells and surface markers of tetramer HCV-NS3, 1292-positive cells during IFN therapy. Solid lines represent viral responders to IFN therapy (sustained viral responders, SVRs) and the broken lines represent transient viral responders (TVRs) whose HCV reappeared within 6 months after the end of IFN therapy. Horizontal broken lines in the upper four panels indicate the cut-off values for HLA tetramers, as mentioned in Materials and methods. The HLA-DR positive rates among tetramer (HCV-NS3, 1292)-positive cells were higher in SVRs than in TVRs at the start of IFN treatment and further increased after 2 weeks of IFN therapy.

TVR) and in the other 3 cases HCV remained below the detection limit (sustained viral responder, SVR) (Fig. 1).

HCV-specific CD8+ T-cell responses during phlebotomy. During phlebotomy, we analyzed HCV-specific CD8+ T-cell responses in 5 cases (patients 1, 3, 4, 5, and 6); and among them, 3 cases were biochemical responders to phlebotomy and the other 2 cases were non-responders (Table I and Fig. 2). The numbers of HLA-A*24 tetramer-positive T-cells were above the cut-off levels for all 3 of the tetramers, HCV-E2.717, HCV-NS3.1292, and HCV-NS4.1760. There was no correlation between the biochemical effects of phlebotomy and the frequencies of tetramer-positive T-cells. We also analyzed the phenotypes of tetramer-positive cells by staining CD45RO, CD27, CD38 and HLA-DR, but we did not observe a correlation between the biochemical effects and the phenotypes. The data indicate that phlebotomy displayed its therapeutic effects for the patients with chronic hepatitis C without affecting the frequencies and phenotypes of HCV-specific CD8+ T-cell responses.

HCV-specific CD8+ T-cell responses during IFN therapy. During and after IFN therapy, we analyzed HCV-specific CD8+ T-cell responses in 2 sustained responders (patients 1 and 5) and 2 transient responders (patients 3 and 6) (Table I and Fig. 3). The three different tetramer-positive T-cells,

HCV-E2.717, HCV-NS3. 1292 and HCV-NS4.1760 were also detectable at levels above the cut-off during IFN therapy. The frequencies of HCV-E2.717 positive cells were higher in SVR patients than in TVR patients before, during and 6 months after IFN therapy. Interestingly, activation marker HLA-DR positive rates among tetramer-positive cells were higher in SVR patients than in TVR patients before IFN treatment and increased further after 2 weeks of IFN therapy and beyond. These results demonstrate that IFN therapy exerted its beneficial effects on the patients with high frequencies of the HCV-specific CD8+ T-cells and enhanced expression of the activation markers, suggesting that the efficacy of IFN therapy for chronic hepatitis C may be mediated by the virus-specific T-cell dependent immunity.

Discussion

For patients with chronic hepatitis C who do not respond to IFN therapy, other treatments to reduce inflammation and decrease transaminase levels are indicated to slow the progression of fibrosis and to lower the incidence of hepato-cellular carcinoma. Phlebotomy is one of the therapies used to decrease the inflammation in the liver. In this study, we analyzed HCV-specific CD8+ T-cell responses in 6 patients with chronic hepatitis C treated by phlebotomy followed by IFN therapy. HLA-A*24 tetramer HCV-E2.717ÅAHCV-

and HCV-NS4.1760 positive T-cells were detected above the cut-off values. During phlebotomy, there was no correlation between the effectiveness of treatments and virological and immunological parameters, such as HCV viral loads, frequencies of tetramer-positive cells and their phenotypes of activation status, although 4 of the 6 cases achieved biochemical improvement. During IFN therapy, interestingly, the frequencies of HCV-E2.717 positive cells were higher in SVR patients than in TVR patients. Additionally, proportions of HLA-DR positive cells among tetramer-positive cells were higher in SVRs than in TVRs at the start of treatment; the proportions increased after 2 weeks of IFN administration and remained elevated during the follow-up periods.

Phlebotomy is thought to be effective by correcting iron excess in chronic hepatitis C. The previous studies have reported that the progression of fibrosis is faster in chronic hepatitis C patients with congenital hemochromatosis (7), that combination therapy with IFN and phlebotomy may be more effective than IFN monotherapy (8), that hemosiderin deposition may be a predictive factor for IFN efficacy (9) and that dietary iron overload may be a risk factor for hepato-cellular carcinoma (10). Inhibition of the toxic effects of superoxide or excess iron has been postulated as a mechanism underlying the therapeutic effects of phlebotomy (11); however, the possible involvement of immunological mechanisms had not been addressed.

Recently developed HLA-class I peptide tetramers, consisting of fluorescently-tagged tetrameric complexes of HLA heavy chains folded around epitope peptides, allow the sensitive and precise enumeration of T lymphocytes with specific T-cell antigen receptors (TCR) (23,24). With regard to HCV infection, this technology revealed that epitope-specific CD8^{+} T lymphocytes are not only detectable in *in vitro* expanded CD8^{+} T lymphocytes but also in freshly isolated PBMCs at more than 10-fold higher frequencies than those previously reported (25). Furthermore, the technology has facilitated the phenotypic, functional and molecular analysis of virus-specific immune responses at the single cell level (25). Additionally, by means of tetramers, the relative frequencies of T lymphocytes specific for different epitopes were observed to change during the course of viral infection (26). We have reported the frequency, phenotype and clinical significance of HCV-specific CD8^{+} T lymphocytes using five different HLA-A*24 tetramers in HCV-infected Japanese patients (17).

Manfras *et al* reported that increased oligoclonality of circulating CD8^{+} T-cells in chronic HCV infection was an indicator of a poor clinical reponse to IFN-α therapy; that IFN-α therapy enhanced the differentiation of CD8^{+} T-cells towards a late differentiation phenotype (CD28^{-} CD57^{+}); and that in cases of virus elimination, there was disappearance of expanded, terminally-differentiated CD8^{+} cells (27). In our study, we found that the HLA-DR positive CD8^{+} T-cells increased after 2 weeks of IFN therapy. On the other hand, during phlebotomy, there was no correlation between the improvement of liver function parameters and the frequencies of tetramer-positive cells or changes in the levels of activation markers. These findings may indicate that the mechanisms of phlebotomy and IFN therapy differ immunologically.

This is the first study to observe the alteration of HCV-specific T-cells, not only during IFN therapy, but also during the phlebotomy and the findings suggest that there may be important differences in their immunological aspects. The use of a combination of therapies which have different but complimentary mechanisms may be more beneficial for the treatment of chronic hepatitis C.

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