best of our knowledge, this is the first demonstration of additive antitumor effects of IFN-α and CpG ODN on DC-based therapy *in vivo*. Our findings suggest that DC-based immunotherapy in combination with CpG ODN and IFN-α gene therapy has potential for inducing potent immune responses and that it would be applicable for clinical antitumor therapy, although further investigations are required.

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Original Article

Magnitude of CD8$^+$ T-cell responses against hepatitis C virus and severity of hepatitis do not necessarily determine outcomes in acute hepatitis C virus infection

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Aim: We investigated the relationship between the magnitude of comprehensive hepatitis C virus (HCV)-specific CD8$^+$ T-cell responses and the clinical course of acute HCV infection.

Methods: Six consecutive patients with acute HCV infection were studied. Analysis of HCV-specific CD8$^+$ T-cell responses was performed using an interferon-γ-based enzyme-linked immunospot assay using peripheral CD8$^+$ T-cells, monocytes and 297 20-mer synthetic peptides overlapping by 10 residues and spanning the entire HCV sequence of genotype 1b.

Results: Five patients presented detectable HCV-specific CD8$^+$ T-cell responses against a single and different peptide, whereas 1 patient showed responses against three different peptides. Neither the magnitude of HCV-specific CD8$^+$ T-cell responses nor the severity of hepatitis predicts the outcome of acute hepatitis. The maximum number of HCV-specific CD8$^+$ T-cells correlated with maximum serum alanine aminotransferase level during the course ($r = 0.841$, $P = 0.036$).

Conclusions: HCV-specific CD8$^+$ T-cell responses were detectable in all 6 patients with acute HCV infection, and 6 novel HCV-specific CTL epitopes were identified. Acute HCV infection can resolve with detectable HCV-specific CD8$^+$ T-cell responses, but without development of antibody against HCV.

Key words: acute hepatitis C, CD8$^+$ T-cell response, cytotoxic T lymphocyte, ELISpot assay, hepatitis C virus, interferon therapy

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection often causes chronic hepatitis, with subsequent progression to cirrhosis and hepatocellular carcinoma. CD8$^+$ cytotoxic T lymphocytes (CTLs) are thought to be a crucial component of the host's immune response to HCV infection.[1] Potent CD8$^+$ T-cell responses are frequently observed in patients with resolved HCV infection.[2] Potent HCV-specific CTL responses and symptomatic hepatitis have been reported as predictors of HCV elimination in acute infection.[3,4] We have demonstrated that human leukocyte antigen (HLA) B44-restricted CTL specific to the HCV core protein (amino acid residues 88–96) exists in the peripheral blood of patients with chronic HCV infection,[5,6] and that the magnitude of CTL response is inversely correlated to the serum HCV load.[7] Other groups have also reported that a potent CTL response against HCV is associated with low viral load in chronic HCV infection.[8,9] In addition, the magnitude of an HLA A*0201-restricted CTL response reportedly shows a strong negative association with viral load in progressive human immunodeficiency virus (HIV) infection.[10]

Since HCV is believed to be a non-cytopathic virus, CTLs are also implicated in the host immunopathogenesis of viral infection.[11] The severity of liver damage may be determined by a balance between the viral load and the strength of host immune responses against HCV. However, immune responses to HCV seem to show little correlation to clinical or histological features,[12] and

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pretreatment HCV-specific immune responses tend to hinder viral clearance by interferon (IFN) therapy[13] in HCV-positive patients with advanced hepatic fibrosis. Further studies on immune responses in HCV infection are thus necessary to clarify their involvement in the immunopathogenesis and outcomes of infection.

Host immune responses against HCV are thought to be suppressed in HCV infection.[14,15] Expression of programmed death-1, one of the molecules implicated in T-cell exhaustion, is up-regulated on HCV-specific CTLs,[16] and the interaction of CD81 with HCV E2 protein inhibits non-specific cytotoxicity mediated by natural killer cells.[17] HCV core and E1 proteins are also reported to inhibit dendritic cell maturation.[18] Furthermore, regulatory T-cells are also implicated in immune suppression in HCV infection.[19,20] HCV itself thus seems highly likely to affect the outcome of acute HCV infection and the results of IFN therapy in patients with chronic HCV infection through suppression of host immune responses against HCV.

Previous studies on HCV-specific CTL responses in acute HCV infection have been performed using only a limited number of specified CTL epitopes. CTL responses observed in those studies may not represent responses against the immunodominant epitopes in individual patients. The present study performed comprehensive analyses of $CD8^+$ T-cell responses against HCV using an IFN-γ-based enzyme-linked immunospot (ELISpot) assay with peripheral $CD8^+$ cells from patients and 297 20-mer synthetic peptides overlapping by 10 residues and spanning the entire HCV sequence of genotype 1b. We investigated the relationship between the magnitude of peripheral $CD8^+$ T-cell responses against the immunodominant peptides identified by the comprehensive analyses and the clinical course in 6 consecutive patients who presented acute HCV infection.

METHODS

Patients

WE ANALYZED 6 consecutive patients who were diagnosed as acute HCV infection at Showa University Hospital in 2004 and 2005. Clinical courses were followed for 8 months to 1.5 years. Diagnosis of acute HCV was based on acute clinical onset of hepatitis, detectable HCV RNA in serum at onset, confirmation of previous negative results for anti-HCV antibody, and exclusion of other causes of liver injury. Serum HCV RNA was detected by Amplicore assay (Roche Diagnostics, Tokyo, Japan) or in-house RT-PCR assay that could detect 10 copies of HCV RNA/mL. Serum HCV RNA levels were determined by Amplicore monitor assay (Roche Diagnostics). HLA class I locus genotypes of peripheral blood mononuclear cells (PBMCs) from patients were determined by the PCR sequencing-based typing method at Mitsubishi Chemical Medicine (Tokyo, Japan). Patients who had HCV persistence for >3 months after onset were treated with either pegylated IFN (PegIFN)-α-2a (Pegasys; Chugai Pharmaceutical, Tokyo, Japan), or PegIFN-α-2b (PegIntron; Schering-Plough, Osaka, Japan) in combination with ribavirin (Rebetol; Schering-Plough). Informed consent was obtained from each patient included in this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethical Committee of Showa University.

Synthetic peptides

As in previous studies,[21,22] 297 20-mer peptides overlapping by 10 residues and spanning the entire HCV sequence were synthesized based on the amino acid sequence of the genotype 1b HCV-J strain (accession no. D90208) as Pepsets (Mimotopes, Melbourne, Vic., Australia). Nineteen peptides in the HCV core region, 19 peptides in the E1 region, 42 peptides in the E2/NS1 region, 21 peptides in the NS2 region, 63 peptides in the NS3 region, 35 peptides in the NS4 region, 44 peptides in the NS5A region, and 54 peptides in the NS5B region were synthesized. A total of 7–10 HCV peptides were pooled in a matrix setting. In addition, 15- or 20-mer peptides overlapping 1 amino acid were synthesized as Pepsets to assess minimal and optimal epitopes. These minimal and optimal epitope peptides were synthesized by and purchased from Mimotopes and were >95% pure.

Preparation of $CD8^+$ cells and monocytes

PBMCs were isolated from heparinized peripheral blood by gradient centrifugation using Ficoll-Paque (Pharmacia-LKB Biotechnology, Uppsala, Sweden). Peripheral $CD8^+$ cells and monocytes were separated from PBMCs using CD8 micro-beads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and a Monocyte Isolation Kit II (Miltenyi Biotec), respectively. These cells were isolated using an autoMACS Pro Separator (Miltenyi Biotec). Purity of these cells was >95% on flow cytometry (data not shown).

ELISpot assay

ELISpot assay was performed using an IFN-γ-based ELISpot assay kit (Mabtech AB, Stockholm, Sweden) as previously described with some modifications.[23] A 96-well microtiter plate with a nitrocellulose membrane bottom (Millititer; Millipore, Bedford, MA, USA) was coated with 100 μL of anti IFN-γ monoclonal antibody at a concentration of 15 μg/mL in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by washing 6 times in Hank's balanced saline solution (HBSS). After blocking with AIM-V medium (Invitrogen Japan, Tokyo, Japan) containing 10% fetal bovine serum, 10^5 $CD8^+$ T-cells, 10^4 autologous monocytes and a peptide mixture or individual peptides at 10 μg/mL each were placed in duplicate and incubated in 100 μL of AIM-V medium at 37°C in a humid atmosphere with 5% CO_2. After incubation for 18 h, the cells were removed by washing the plate 8 times with PBS. Next, 100 μL of biotin-conjugated monoclonal antibody was added to each well, and plates were incubated further for 2 h at room temperature. Wells were washed five times with PBS and incubated with 100 μL streptavidin-alkaline phosphatase for 2 h. Unbound antibodies were removed by washing six times with PBS. Then, 100 μL of alkaline phosphatase substrate (Bio-Rad Laboratories; Richmond, CA, USA) was added to each well and incubated until dark spots emerged. Color development was stopped by washing three times with water, and plates were allowed to dry. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted. Numbers of HCV antigen-specific SFCs were calculated by subtracting the mean number of SFCs from two control wells (without stimulus) from the mean number of SFCs from 2 wells stimulated by HCV antigens. Results were considered as positive when SFCs numbered >10 and the count was 2-fold greater than the control. When HCV-specific $CD8^+$ T-cell responses were analyzed in normal subjects, we were unable to detect any responses to HCV peptides by ELISpot assay (data not shown).

Statistical analyses

Correlations between the 2 groups were studied by linear regression analysis.

RESULTS

Characterization of patients with acute HCV infection

THE CHARACTERISTICS OF 6 consecutive patients with acute HCV infection are shown in Table 1. HCV infection was self-limited in 3 patients, but the remaining 3 patients could not eliminate HCV within 3 months of onset and were successfully treated using PegIFN-α-2a alone (Patients 1 and 2) or PegIFN-α-2b in combination with ribavirin (Patient 3). Although a previous report found that no asymptomatic patients with acute HCV showed self-limited disease,[24] one of our patients with asymptomatic acute hepatitis C (Patient 6) eliminated HCV spontaneously.

HCV-specific $CD8^+$ T-cell responses in patients with acute HCV infection

All patients presented detectable HCV-specific $CD8^+$ T-cell responses by ELISpot assay (Table 2; Fig. 1). Six minimal and optimal epitopes for T-cells were determined using peptides overlapping 1 amino acid, and all 6 epitopes turned out to be novel HCV-specific CTL epitopes (Table 3).[25] Most of these epitopes were within

Table 1 Characteristics of patients with acute hepatitis C virus infection

Patient	Age (years)	Sex	Possible cause of infection	Symptom	Period between cause and onset†	Period between onset and visit‡
1	36	F	unknown	jaundice	–	3 weeks
2	51	F	needle stick	anorexia, jaundice	6 weeks	0 week
3	54	F	unknown	flu-like	–	6 weeks
4	48	F	unknown	jaundice	–	2 weeks
5	29	F	sexual contact	jaundice	unclear	2 weeks
6	27	M	eye exposure	none	8 weeks	0 week

†Period between cause of hepatitis (if applicable) and onset of hepatitis; ‡Period between onset of hepatitis and first visit to our hospital.
F, female; IFN, interferon; M, male.

Table 2 Laboratory findings and results of ELISpot assay

Patient	Max. HCV RNA (kIU/mL)	HCV genotype	Max ALT (IU/L)	Max. T. Bil. (mg/dL)	Outcome	Max. SFCs (/10^5 $CD8^+$ cells†)	HCV peptide recognized by CTLs
1	352	1b	853	14.7	IFN therapy	57	NS3 1367-1386
2	1150	2b	1652	7.0	IFN therapy	386	E2 604-623
3	2800	1b	860	1.7	IFN therapy	282	NS5A 2284-2303
4	<5 (positive)	could not be determined	1085	11.6	self-limited	103	NS4 1758-1777
						214	NS5B 2551-2570
						81	NS5B 2801-2820
5	39	1b	733	5.8	self-limited	114	NS4 1958-1877
6	<5 (positive)	could not be determined	236	0.6	self-limited	44	NS3 1637-1656

†Results are shown as maximum number during the course.
ALT, alanine aminotransferase; CTLs, cytotoxic T lymphocytes; SFCs, spot-forming cells; T. Bil., total bilirubin.

non-structural regions (Table 2; Fig. 1). HLA class I restriction of recognition of the 6 epitopes by $CD8^+$ T-cells could not be determined due to failure of establishment for HCV-specific T-cell lines. Although one patient showed $CD8^+$ T-cell responses against three different epitope peptides, the others only demonstrated responses against single, different peptides.

$CD8^+$ T-cell responses were low at the peak of serum ALT levels in all patients, increased following the peak period, and then decreased except for in 1 patient in whom levels could only be followed for a short time. Two of the 3 patients with self-limited HCV infection (Patients 4 and 5) presented strong $CD8^+$ T-cell responses (>100 SFCs/10^5 $CD8^+$ cells) to HCV peptides and overt jaundice (Fig. 1; Patients 4 and 5). However, the remaining patient with self-limited hepatitis (Patient 6) presented with relatively weak HCV-specific $CD8^+$ T-cell responses and was asymptomatic (Fig. 1; Patient 6). Patient 6 was a trainee in medicine, and HCV-contaminated blood splashed into his eye when he attended the surgery of a patient with chronic HCV infection. After exposure, he was followed every 4 weeks for possible HCV infection. Although he was asymptomatic, serum ALT levels were increased 8 weeks after exposure. Although serum HCV RNA was not detected using a commercially available Amplicore assay, peripheral $CD8^+$ T-cells from the patient presented a weak but definite positive response to one of the HCV peptides. Repeated assay of serum HCV RNA by in-house RT-PCR yielded positive results, suggesting that the serum HCV RNA level was within the range of 10–50 copies/mL. Serum HCV RNA was positive by in-house RT-PCR for a very short time. Although the patient persistently presented with a positive HCV-specific $CD8^+$ T-cell response, anti-HCV antibody remained negative and did not develop eventually.

Two of the 3 patients who required IFN therapy to eradicate HCV showed vigorous HCV-specific $CD8^+$ T-cell responses, while the remaining patient presented a weak response. In all patients, peripheral HCV-specific $CD8^+$ T-cell responses tended to decrease as serum HCV levels decreased. In Patients 2 and 3, early changes of HCV-specific $CD8^+$ T-cell responses in IFN therapy were analyzed in detail. In both HCV-specific $CD8^+$ T-cell responses were enhanced after a week of IFN therapy, then were reduced within 1 month of starting therapy (Fig. 2a,b).

HCV-specific $CD8^+$ T-cell responses persisted even after clearance of HCV, although at a low level, both in patients with self-limited acute hepatitis and in those with IFN-treated hepatitis.

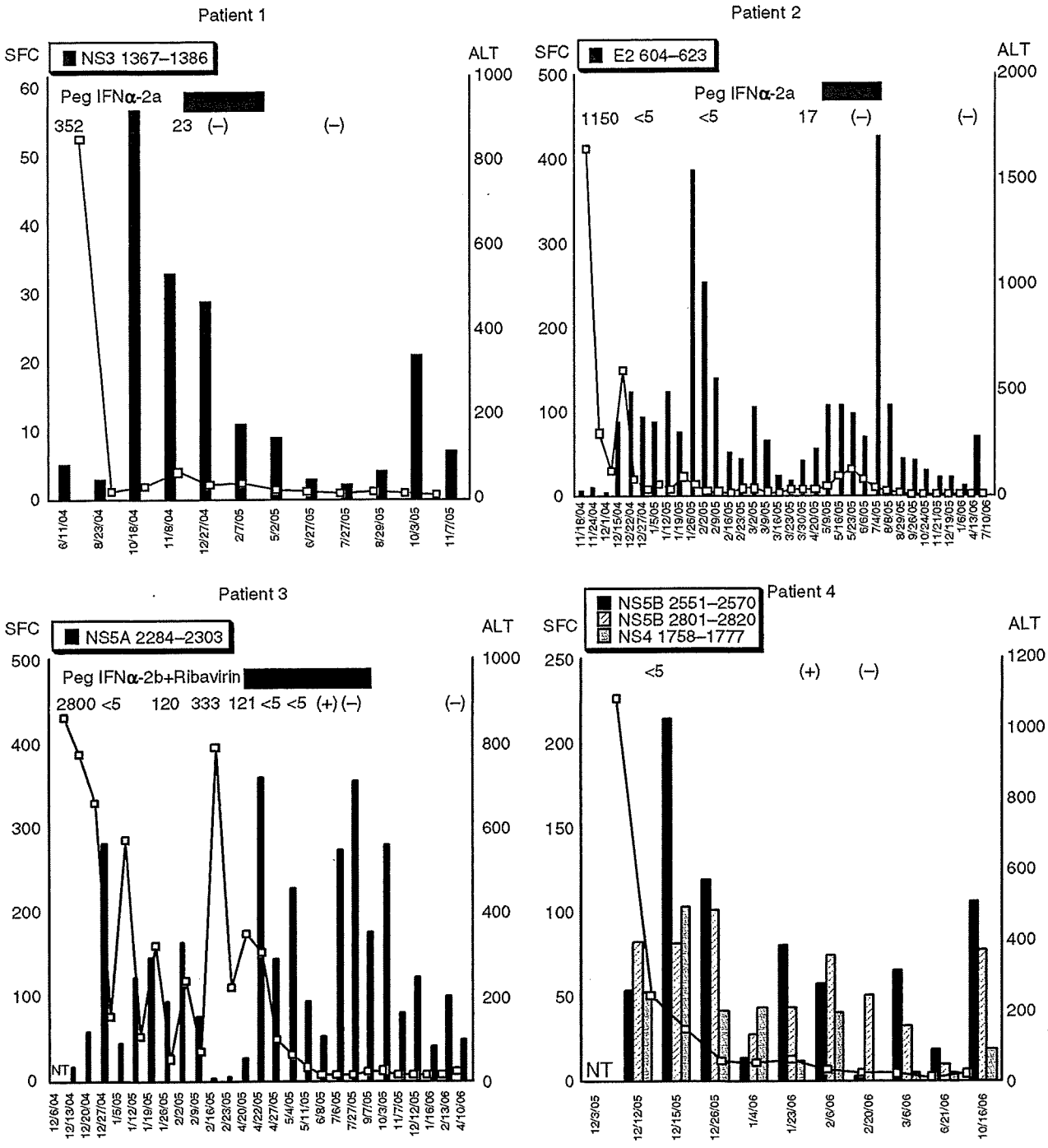

Figure 1 Clinical courses of 6 patients with acute hepatitis C virus (HCV) infection. The results show serum ALT level (IU/L; line) and the number of spot-forming cells (SFCs, /10^5 $CD8^+$ cells; bar) stimulated by the indicated peptides. The numbers displayed in the upper side of each graph show the titer of HCV RNA (kIU/mL). When HCV RNA was not detected, the result was displayed as (–). The period of IFN therapy is displayed as a closed square at the upper side of the graph if the patient was treated. NT, not tested.

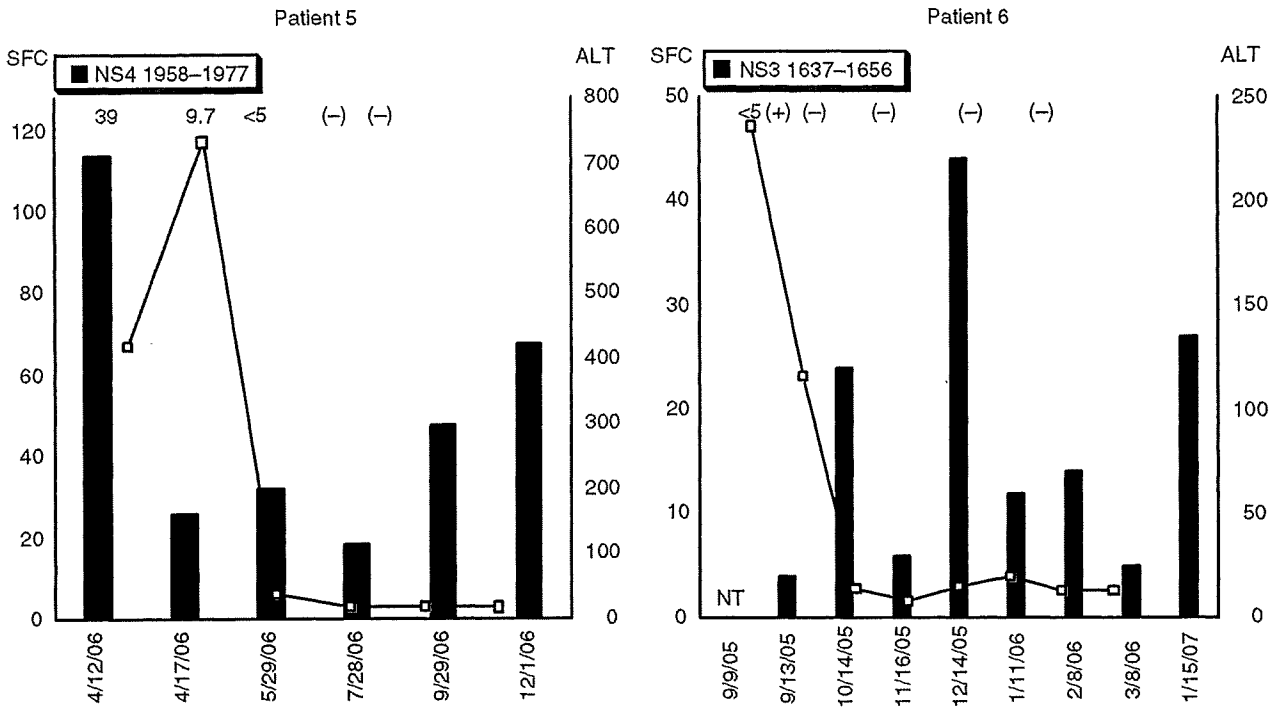

Figure 1 *Continued*

Magnitude of HCV-specific CD8⁺ T-cell responses and outcomes of acute HCV

No clear relationship between the outcome of acute HCV and the magnitude of HCV-specific CD8⁺ T-cell response was observed. The severity of clinical symptoms and maximum serum HCV RNA levels were also not correlated with peripheral CD8⁺ T-cell responses. However, the maximum number of HCV-specific CD8⁺ T-cells correlated with maximum serum ALT levels ($r = 0.841$, $P = 0.036$) (Fig. 3).

Table 3 Peptides detected by comprehensive ELISpot assay, and minimal and optimal epitopes

Patient	HLA	Peptides recognized by CD8+ T cells	Minimal and optimal epitope†
1	A*2602, 3101, B*5101, 5102, C*1402, 1502	LSNTGEIPFYGKAIPIEAIK (NS3 1367–1386)	IPFYGKAI (NS3 1373–1380)
2	A*0402, B*0702, 5201 C*0702, 1202	TPRCLVDYPYRLWHYPCTIN (E2 604–623)	YPYRLWHY (E2 611–618)
3	A*1101, 3101, B*6701, 5101 C*0702, 1402	ALPIWARPDYNPPLLESWKS (NS5A 2284–2303)	RPDYNPPLL (NS5A 2290–2298)
4	A*2402, B*5201, C*1202	EAFWAKHMWNFISGIQYLAG (NS4 1758–1777)	AFWAKHMWNF (NS4 1759–1768)
		TPIDTTIMAKNEVFCVQPEK (NS5B 2551–2570)	TIMAKNEVF (NS5B 2556–2564)
		YYLTRDPTTPLARAAWETVR (NS5B 2801–2820)	LTRDPTTPL (NS5B 2803–2811)
5	A*0201, 0301, B*4402, 4601, C*0102, 0501	KRLHQWINEDCSTPCSGSWL (NS4 1958–1977)	not determined
6	A*2402, 4801, B*5201 C*0803, 1202	LTHPITKFVMACMSADLEVV (NS3 1637–1656)	not determined

†Minimal and optimal epitopes were assessed using 15-mer or 20-mer peptides overlapping 1 amino acid synthesized as Pepsets, and were confirmed using synthesized epitope peptides by the ELISpot assay.
HLA, human leukocyte antigen.

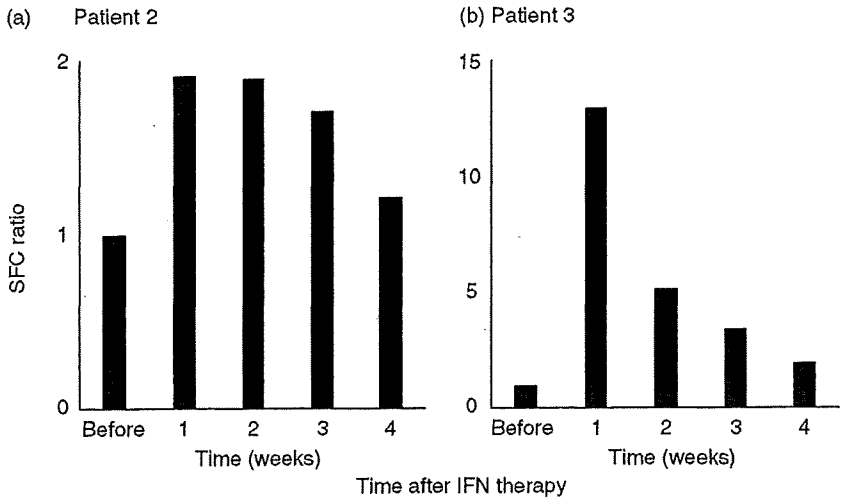

Figure 2 IFN therapy temporarily enhanced HCV-specific CTL response in 2 of 3 patients with acute hepatitis C virus (HCV). The result shows the comparison of HCV-specific cytotoxic T lymphocyte (CTL) responses between before and after IFN therapy in patients with acute hepatitis C (A, Patient 2; B, Patient 3). Spot-forming cell (SFC) ratios were determined using the formula: number of SFCs after IFN therapy/number of SFCs before therapy.

DISCUSSION

WE HAVE PREVIOUSLY reported that HCV-specific and HLA B44-restricted CTL response responding to HCV core amino acid residues 88–96 is inversely correlated with serum HCV viral load in patients with chronic HCV.[7] The HLA B44-restricted CTL seemed immunodominant, as CTLs restricted to other HLAs could barely be induced by the same procedure. The function of HCV-specific CTLs as effector cells has been reported to be impaired in HCV infection.[26–29] Since immune responses against HCV are implicated in the injury of hepatocytes as well as in host defenses, monitoring CTL responses against HCV in patients with acute HCV infection would contribute to clarification of the pathogenesis of HCV as well as the development of new treatment strategies against HCV infection.

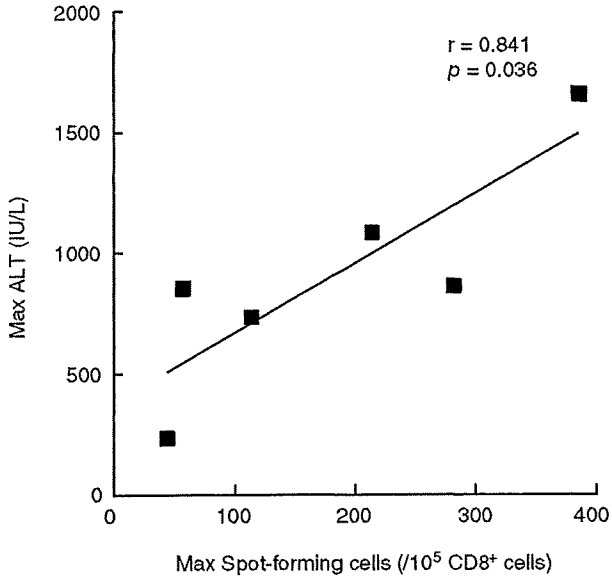

Figure 3 The maximum number of HCV-specific cytotoxic T lymphocyte (CTLs) correlated with the maximum serum ALT level, but not with maximum total bilirubin during the course. The results show the relationship between HCV-specific CTL response and serum ALT level. The line displayed in the figure reveals a regression curve.

The present study performed comprehensive CTL analysis using an IFN-γ-based ELISpot assay with peripheral $CD8^+$ T-cells from patients, autologous monocytes as antigen-presenting cells, and peptides spanning the whole HCV protein to study CTL responses in patients with acute HCV infection. Analyses of CTL responses restricted by a few, known HLA alleles would have shown only limited immunological information. The advantage of comprehensive ELISpot assay is the ability to detect all CTL responses against the whole HCV protein in every patient with individual HLA alleles.

An HCV-specific CTL response was detected in all patients who suffered from acute HCV infection, although the magnitude of the CTL response differed in each patient. All patients presented $CD8^+$ T-cell response against a single and different epitope, with the exception of the one patient with responses against 3 epitopes, and 6 were novel epitopes.

Our results differ from a previous report that observed multispecific CTL responses against HCV in patients with acute HCV infection that spontaneously resolved.[2] Although that study also used ELISpot assay, the

methods differed from our own, with whole PBMCs used for stimulation with HCV proteins as opposed to our use of isolated CD8+ T-cells. Their method may have been more efficient at stimulating CTLs, although background levels were also high. Otherwise, the difference may be due to detection threshold used and we might have picked up only strongly immunogenic antigens.

Three of the 6 patients with acute HCV who needed IFN therapy and potent HCV-specific CD8+ T-cell responses did not eliminate HCV spontaneously. Symptoms such as jaundice have been reported as a surrogate marker of potent immune response and tendency to eradicate HCV without therapy.[30,31] However, 2 of 4 patients with overt jaundice in this study needed IFN therapy to eradicate HCV. Our results suggest that the magnitude of cytotoxic T-cell responses and the severity of hepatitis are not necessarily predictive of the outcome of acute HCV infection as reported previously,[2] although CD8 responses have been suggested to determine the outcome of acute HCV.[32]

The magnitude of HCV-specific CD8+ T-cell responses in peripheral blood correlated well with serum ALT levels and severity of hepatic inflammation, although only a small number of patients were analyzed. Although patients with acute HCV showing multispecific CTL responses display relatively low-grade severity of hepatitis,[33] our only patient with multispecific CTL responses presented with the highest serum ALT level and deep jaundice. The frequency of HCV-specific CD8+ T-cells was low at the peak serum ALT level in every patient. A potent HCV-specific CD8+ T-cell response tended to be detected within 1 month after the elevation of serum ALT level.

A previous report showed the difference between liver-infiltrating lymphocytes and peripheral lymphocytes in a chimpanzee model.[34] That finding suggests that HCV-specific CD8+ T-cells are compartmentalized in the liver at the peak of hepatic inflammation and are released into or stay in the peripheral blood as inflammation subsides. In chronic HCV, CD8+ T-cells in the liver are associated with serum ALT level.[8] Although HCV-specific CTLs injure hepatocytes infected with HCV rather than act as protection against HCV in chronic HCV infection, this action prevents the expansion of HCV infection in the early phase of acute HCV.

IFN therapy temporarily enhanced HCV-specific CD8+ T-cell response at 1 week after starting therapy, but this response decreased within a month. This observation is consistent with previous reports.[3,32,35] Diminished serum HCV RNA level might result in reduced CTL response within several weeks, and eradication of HCV did not depend on the magnitude of HCV-specific CTL responses. HCV-specific CD8+ T-cell responses remained after the elimination of HCV in both patients with self-limited and IFN-treated patients, although at diminished levels. This contrasts with our previous finding that HCV-specific CD8+ T-cell response dropped to undetectable or very low levels with treatment and did not improve after completion of treatment.[23] Early treatment might lead to the maintenance of HCV-specific CD8+ T-cell response, and may explain that early treatment could eradicate HCV in almost all patients with acute HCV.

As described above, some contradictions exist between our study and previous reports. We assessed only IFN-γ production by CD8+ cells using ELISpot assay. Further analyses including functional assay of CTLs, such as investigation of the cytotoxic activity of these cells, will thus be required. Regulation of CTLs (e.g. analysis of regulatory T-cells) seems crucial to understanding the immunopathogenesis of HCV infection. We believe that the outcome of HCV infection depends on the sum of immune responses induced in the host, including not only innate and acquired immune responses, but also regulatory T-cells. The magnitude of HCV-specific CD8+ cell responses is thus unable to predict the outcome of HCV infection, although HCV-specific CD8+ cells would contribute to HCV eradication.

We described one patient who eliminated HCV without subsequent development of antibody against HCV. At 8 weeks after exposure to HCV, he displayed an HCV-specific CD8+ T-cell response and serum HCV RNA was estimated to be within a range of 10–50 copies/mL. We assume that innate immunity effectively suppressed the outgrowth of HCV under a low level and CTL, when induced, quickly eradicated HCV with minimal damage to the liver without development of antibody against HCV. Our observation is consistent with the report that some individuals working in a medical biochemistry laboratory presented with HCV-specific CTL responses without the presence of antibody to HCV and HCV RNA in sera.[36] To the best of our knowledge, this is the first report to present the case of a patient with self-limited acute HCV infection displaying HCV-specific CD8+ T-cell responses, but not developing anti-HCV antibody. HCV infection and spontaneous resolution may thus be more common than previously thought.

In conclusion, the magnitude of CD8+ T-cell responses against HCV seems to correlate with the severity of acute HCV, but does not necessarily predict outcome. Patients with acute HCV infection may elimi-

nate the virus with detectable $CD8^+$ T-cell responses, but without development of antibody against HCV.

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Infection of B Cells With Hepatitis C Virus for the Development of Lymphoproliferative Disorders in Patients With Chronic Hepatitis C

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Infection with hepatitis C virus (HCV) is associated with lymphoproliferative disorders, represented by essential mixed cryoglobulinemia and B-cell non-Hodgkin's lymphoma, but the pathogenic mechanism remains obscure. HCV may infect B cells or interact with their cell surface receptors, and induce lymphoproliferation. The influence of HCV infection of B cells on the development of lymphoproliferative disorders was evaluated in 75 patients with persistent HCV infection. HCV infection was more prevalent (63% vs. 16%, 14%, or 17% $P < 0.05$ for each), and HCV RNA levels were higher (3.35 ± 3.85 vs. 1.75 ± 2.52, 2.15 ± 2.94 or 2.10 ± 2.90 log copies/100 ng, $P < 0.01$ for each) in B cells than $CD4^+$, $CD8^+$ T cells or other cells. Negative-strand HCV RNA, as a marker of viral replication, was detected in B cells from four of the 75 (5%) patients. Markers for lymphoproliferative disorders were more frequent in the 50 patients with chronic hepatitis C than the 32 with chronic hepatitis B, including cryoglobulinemia (26% vs. 0%, $P < 0.001$), low CH_{50} levels (48% vs. 3%, $P = 0.012$), and the clonality of B cells (12% vs. 0%, $P < 0.01$). By multivariate analysis, HCV RNA in B cells was an independent factor associated with the presence of at least one marker for lymphoproliferation (odds ratio: 1.98 [95% confidence interval: 1.36–7.24], $P = 0.027$). Based on the results obtained, the infection of B cells with HCV would play an important role in the development of lymphoproliferative disorders. ***J. Med. Virol. 81:619–627, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: B cells; clonality; cryoglobulinemia; hepatitis C virus; lymphoproliferative disorders

INTRODUCTION

Hepatitis C virus (HCV) can induce chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [Tong et al., 1995; Ikeda et al., 1998]. In addition, some patients infected with HCV develop proliferative disorders of lymphocytes, such as mixed cryoglobulinemia [Agnello et al., 1992; Frangeul et al., 1996; Donada et al., 1998] and B-cell non-Hodgkin's lymphoma (NHL) [Ferri et al., 1994]. Cryoglobulinemia represents the oligoclonal proliferation of B cells and occurs in 19–56% of patients infected with HCV [Mazzaro et al., 1996; Donada et al., 1998; Weiner et al., 1998; Schmidt et al., 2000], while antibody to HCV (anti-HCV) and HCV RNA are detected more frequently in the patients with non-Hodgkin's lymphoma than in the general population (30% vs. 1.3%) [Ferri et al., 1994]. On the basis of these observations, cryoglobulinemia is considered to be a marker for lymphoproliferative disorders. In addition, rheumatoid factor (RF) in high titers and hypocomplementemia (low levels of C3, C4, or CH_{50}) are regarded as immunological markers for autoimmune disease and lymphoproliferation [Ramos-Casals et al., 2005]. In the patients with Sjögren's syndrome, for instance, hypocomplementemia was closely associated with the development of lymphoma [Ramos-Casals et al., 2005].

Although an epidemiological association has been noted between HCV infection and lymphoproliferative disorders, the pathogenic mechanisms underlying it have remained unclear. HCV would infect B cells persistently, and induce somatic mutations toward propagation in them. Recently, the replication of HCV was demonstrated in a B-cell line established from a patient infected with HCV [Sung et al., 2003], and

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somatic hypermutations in the immunoglobulin genes as well as proto-oncogenes were observed in a B-cell line infected with HCV [Machida et al., 2004]. These observations suggest direct and/or indirect effects of HCV infection of B cells on the induction of lymphoproliferative disorders. HCV infection by itself or stimulation by immune complexes containing viral antigens may trigger the clonal proliferation of B cells for the development of lymphoproliferative disorders in patients with chronic hepatitis C [Agnello, 1995; Ivanovski et al., 1998].

There is some evidence for the replication of HCV in peripheral blood mononuclear cells (PBMCs) of patients who are infected with HCV persistently [Moldvay et al., 1994; Lerat et al., 1998; Zignego and Brechot, 1999], although it is not known which of the T-, B-, and other cell-types supports the replication of HCV. It may replicate at very low levels in lymphoid cells, and they may serve as an extra-hepatic reservoir; this is implicated in recurrence and persistence of HCV infection in immunosuppressed individuals [Laskus et al., 2000]. Although current antiviral therapies can eliminate HCV from the serum, it may survive in lymphocytes thereafter [Pham et al., 2004; Radkowski et al., 2005]. The efficacy of interferon therapy on the HCV infection of lymphocytes is, as yet, uncertain.

The frequency of HCV infection of peripheral blood mononuclear cells (PBMCs) was examined in 75 patients with chronic hepatitis C, and HCV RNA levels were determined in $CD4^+$, $CD8^+$ T-cells, B-cells, and other cells. The replication of HCV in B cells was correlated subsequently with lymphoproliferative disorders.

MATERIALS AND METHODS

Patients

During 2003 through 2006, 75 patients infected with HCV were admitted to the Showa University Hospital, including two with acute hepatitis, 50 with chronic hepatitis, two with liver cirrhosis, 16 with hepatocellular carcinoma and five with non-Hodgkin's lymphoma. The diagnosis of HCV infection was confirmed in all these patients by the detection of anti-HCV and HCV RNA in the serum. All the patients were negative for hepatitis B surface antigen (HBsAg) or antibody to human immunodeficiency virus type-1. Serving as controls were 28 healthy individuals without HCV infection and 32 patients with chronic hepatitis B who did not have liver cirrhosis, hepatocellular carcinoma or non-Hodgkin's lymphoma. Informed consent was obtained from every participant for the purpose of this study, and the study was approved by the Ethics Committee of Showa University School of Medicine.

Isolation of Lymphoid Cells

PBMCs ($2.83 \pm 1.46 \times 10^7$) were obtained from whole blood (30 ml) by the centrifugation in a Ficoll/Hypaque gradient. Beads with the affinity for $CD8^+$ cells (MicroBeads®; Miltenyi Biotte, Bergisch Gladbach, Germany) were added to PBMCs, and the cell suspension was mixed well, incubated for 15 min at 4°C and centrifuged at 900*g* for 10 min in a tube. The tube was then placed on a magnet, and the supernatant floating free cells was transferred to another tube. The pellet containing $CD8^+$ cells was collected and stored at −80°C until use. $CD4^+$ and $CD19^+$ cells were separated from the supernatant using similar procedures. The remaining supernatant was pelleted to make the 'other cell' fraction. Each compartment of PBMCs contained $\sim 11 \times 10^5$–10^6 cells.

Quantitation of HCV RNA in Lymphoid Cells

HCV RNA was determined by reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was extracted from each cellular compartment using Trizol® Reagent (Invitrogen, Carlsbad, CA). The RNA solution was stirred-up, and a portion (1 μl from the total of 50 μl) was reverse-transcribed by AMV® RT (Roche, Mannheim, Germany) and amplified by the single-step PCR for 40 cycles with appropriate primers (5′-CGC GCG ACT AGG AAG ACT TC-3′ and 5′-ATA GAG AAA GAG CAAC CA GG-3′) that are complementary to the 5′-UTR sequence. HCV RNA was determined in 100 ng of each RNA sample by the real-time RT-PCR using the primers described previously [Ito et al., 2001]; it has a detection range over 1.0–8.0 log copies. HCV RNA was recorded as positive for the samples with titers exceeding 1.0 log copies/100 ng, in order to exclude contamination of lymphoid cells with serum HCV RNA.

RT-PCR for Detecting Negative-Strand HCV RNA

Negative-strand HCV RNA was determined by the strand-specific RT-PCR. A portion of total RNA (1 μl from 50 μl) extracted from the B-cell fraction (10^5 cells) was reverse-transcribed with the sense primer (5′-AGA CAT CGG GCC AGA AGT GTC C-3′) complementary to a partial NS5B sequence of negative-strand HCV RNA, and amplified by the hot-started single-step RT-PCR for 40 cycles using the GeneAmp® EZ rTth RNA PCR kit (Applied Biosystems, Branchburg, CA) with the same primer and an antisense primer (5′-CGT TCA TCG GTT GGG GAG CAG G-3′) located downstream of it [Castillo et al., 2005]. Controls were negative- and positive-strand HCV RNA species that had been generated by in vitro transcription using the pCVJ4L6S plasmid [Yanagi et al., 1998]. Assays were performed in duplicate for each sample.

Serum Markers of Lymphoproliferative Disorders

Cryoglobulinemia was detected by a semi-quantitative centrifugation method. Briefly, blood samples were centrifuged at 600*g* for 20 min at 37°C. Sera were cooled to 4°C and left to stand for 48 h, and centrifuged again at 2,500*g* for 10 min at 4°C. The emergence of cryocrit at 4°C and its disappearance by warming up to 37°C for

20 min was regarded positive for cryoglobulin. RF was determined by the latex turbidimetric assay, and C4 and CH_{50} activities by nephelometry and Mayer's method, respectively. Markers of lymphoproliferative disorders were determined in 50 patients with chronic hepatitis C and 32 patients with chronic hepatitis B who had HBsAg in the serum. None of the patients with chronic hepatitis suffered from acute hepatitis, cirrhosis or non-Hodgkin's lymphoma.

Amplification of the V_H Region in Immunoglobulin by PCR

RNA (1 μl) from PBMCs or B cells was reverse-transcribed into cDNA and amplified using the GeneAmp® EZ rTth RNA PCR kit (Applied Biosystems) in accordance with the manufacturer's instructions. Amplification was carried out with FW1 primer (5′-AGG TGC AGC TGG A[T]GG[C] AGT C[G]T[G]G G-3′) in the V_H region and hM3 primer (5′-GGA AAA GGG TTG GGG CGG AT-3′) located 8 nt downstream from the start of C_H1 exon in Cμ region. PCR products were visualized by staining with ethidium bromide after they had been electrophoresed on 1% agarose gels.

Fingerprinting Assay for the Ig Gene

The clonality of B cells was examined by the fingerprinting assay specific for isotypes of the immunoglobulin (Ig) gene by the method of Ivanovski et al. [1998]. Briefly, the PCR products of the Ig-V_H gene, from 75 patients infected with HCV and 28 healthy controls, were examined for the length of the complementarity-determining region 3 (CDR3) by primer extension using a primer (hFW3; 5′-CTG AGG ACA CGG CCG TGT ATT ACT G-3′) complementary to a conserved sequence in human V_H framework 3 (FW3) regions. The hFW3 primer was end-labeled with [γ-^{32}P]ATP (150 mCi/ml; Japan Radioisotope Association, Tokyo, Japan) using the phage T4 polynucleotide kinase (T4PNK; Takara, Shiga, Japan). The reaction mixture (18 μl), containing PCR products, 1 μM ^{32}P-labeled primer, 25 mM dNTP, 10× PCR buffer and 1 unit of Taq polymerase, was subjected to denaturation at 95°C for 8 min, annealing at 64°C for 1 min and extension at 72°C for 15 min. The reactant (9 μl) was separated by electrophoresis on 6% polyacrylamide gel supplemented with 6 M urea, dried and exposed onto radiographic films.

Subcloning and Sequencing of the V_H Region Gene

One PCR product from a patient infected with HCV that exhibited oligoclonal bands and another from a control subject, without displaying any clonal band on the Ig fingerprinting assay, were purified on gel-electrophoresis and sub-cloned into the pCR-TOPO vector (Invitrogen, Leek, The Netherlands). After they had transformed *Escherichia coli* (DH5α), 26 and 16 clones from the patient and control, respectively, were selected and the V_H region was sequenced using the Big Dye® Terminator ver.1.1 Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan). Deduced amino acid sequences of the VDJ region of IgM heavy chain were aligned by the BLAST search, and analyzed for any homology among clones by the GENETYX-Mac ver.13.0 software (Genetyx, Tokyo, Japan).

Statistical Analysis

The median of continuous variables, without the normal distribution, was compared by the Mann–Whitney *U* test. Comparison of discontinuous variables was performed by the χ^2 test or Fisher's exact test. A P value < 0.05 was considered statistically significant. Values with the normal distribution were expressed as the mean ± SD. Data of variables, not distributed normally, were transformed into log values as required. Logistic regression modeling was used in the multivariate analysis for association with lymphoproliferative disorders with the JMP ver. 7 software (SAS Institute, Cary, NC).

RESULTS

Detection of HCV RNA in B Cells

Table I compares the detection of HCV RNA in various cellular compartments. HCV RNA was detected more frequently in B cells than $CD4^+$, $CD8^+$ T cells or other cells from the 75 patients (63% vs. 16%, 14%, or 17%, $P < 0.05$ for each). There were no significant differences in the detection of HCV RNA in lymphoid cells between the patients with chronic hepatitis and those with hepatocellular carcinoma or non-Hodgkin's lymphoma. HCV RNA was detected in B cells from 55 (73%) patients by the conventional RT-PCR at screening. Eight of them, with HCV RNA titers <1.0 log copies/100 ng RNA by the real-time RT-PCR, were deemed negative for HCV RNA

TABLE I. Frequency of HCV RNA in Diverse Cell Compartments From Patients Infected With HCV

Cell types	Total (n = 75)	Chronic hepatitis[a] (n = 54)	Non-Hodgkin lymphoma (n = 5)	Hepatocellular carcinoma (n = 16)
$CD8^+$ T cells	12 (16%)*	6 (11%)*	2 (40%)	4 (25%)
$CD4^+$ T cells	11 (15%)*	6 (11%)*	3 (60%)	2 (13%)
B cells	47 (63%)	32 (59%)	3 (60%)	12 (75%)
Others	17 (23%)*	11 (20%)**	3 (60%)	3 (19%)

[a]Two patients each with acute hepatitis and cirrhosis without hepatocellular carcinoma were included.
*Significantly lower than the detection in B cells ($P < 0.05$).
**Significantly lower than the detection in B cells ($P < 0.01$).

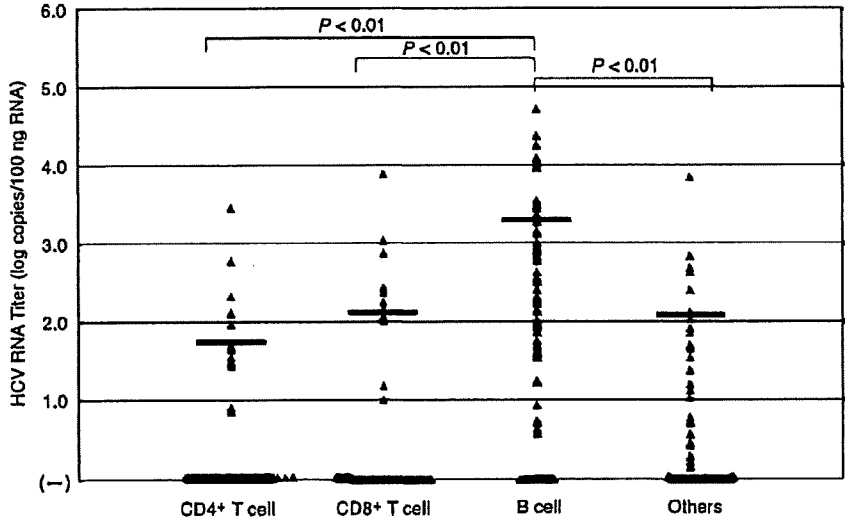

Fig. 1. HCV RNA titers in various compartments of lymphoid cells. Bold bars indicate mean values.

in order to exclude contamination by serum HCV RNA. The distribution of HCV RNA titers in various cell compartments is illustrated in Figure 1. HCV RNA levels were higher in B cells than in $CD4^+$, $CD8^+$ T cells or other cells (3.35 ± 3.85 vs. 1.75 ± 2.52, 2.15 ± 2.94 or 2.10 ± 2.90 log copies/100 ng, $P < 0.01$ for each).

Replication of HCV in B Cells

A method for detecting minus-strand HCV RNA was developed (see Materials and Methods Section). It could detect negative-strand HCV RNA specifically with a sensitivity of 3.0 log copies/ml (range: 3.0–6.0 log copies/ml) without a self-priming of positive strands in control transcripts (Fig. 2A). None of the 75 patients had >7.0 log copies of HCV RNA in B cells (Fig. 1), thereby indicating the capability of this method to detect negative-strand HCV RNA in the patients studied. Since this assay could not detect <3.0 log copies/ml of negative-strand HCV RNA, which were present in lower titers than positive strands usually, only the 16 patients with HCV RNA in B cells in titers ≥3.0 log copies/ml were analyzed. Negative-strand HCV RNA was detected in four (5%) of the 75 patients, indicating that HCV replicated efficiently in the B cells (lanes 2, 14, 15, and 17 in Fig. 2B).

Markers for Lymphoproliferative Disorders in Patients With Chronic Viral Hepatitis

Table II compares the detection of markers for lymphoproliferative disorders between the 50 patients with chronic hepatitis C and the 32 patients with chronic hepatitis; they did not have acute hepatitis, liver cirrhosis, hepatocellular carcinoma or non-Hodgkin's lymphoma. Cryoglobulinemia and low CH_{50} levels

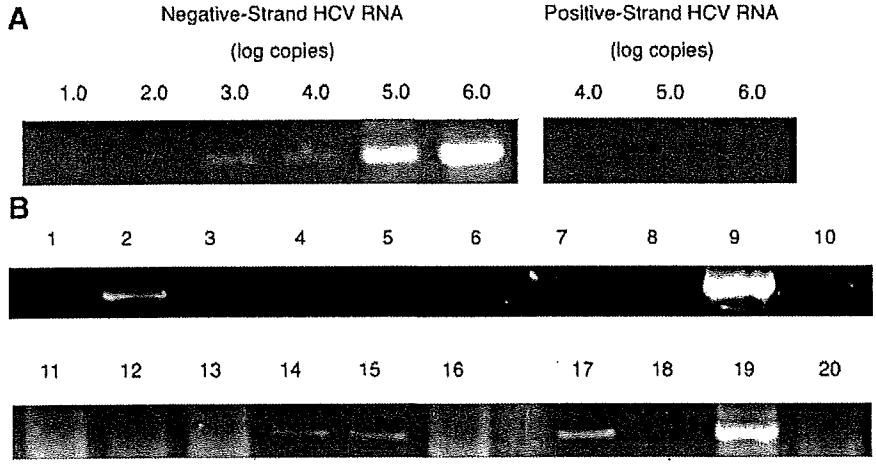

Fig. 2. **A**: Ethidium bromide staining of products of strand-specific RT-PCR for negative- and positive-strand HCV RNA. **B**: Strand specific RT-PCR for negative-strand HCV RNA from B cells of 16 patients. Positive control for negative-strand HCV RNA (**lanes 9** and **19**) and negative control for positive-strands HCV RNA (**lanes 10** and **20**) are included.

TABLE II. Markers for Lymphoproliferative Disorders in Patients Chronic Hepatitis C or B

Features[a]	Hepatitis C (n = 50)	Hepatitis B (n = 32)	Differences *P* value
Age (years)	52.3 ± 11.8	53.9 ± 11.9	NS
Men	32 (64%)	17 (53%)	NS
ALT (IU/L [5–25][b])	96.5 ± 145.9	50.7 ± 79.8	0.008
Cryoglobuminemia	13 (26%)	0 (0%)	<0.001
RF > 10 IU/ml	24 (48%)	13 (41%)	NS
C4 < 10 mg/dl	1 (2%)	1 (3%)	NS
CH_{50} < 20 U/ml	24 (48%)	1 (3%)	0.012
Clonality	6 (12%)	0 (0%)	<0.001
Any marker for lymphoproliferative disorders	37 (74%)	13 (41%)	0.015

ALT, alanine aminotranferase; RF, rheumatoid factor; NS, not significant.
[a]Data are number (%) or the mean ± SD.
[b]Interquartile normal range.

(<20 U/ml) were significantly more frequent in the patients with chronic hepatitis C than hepatitis B, while high RF titers (>10 IU/ml) were common in them both. At least one marker for lymphoproliferative disorders was detected more frequently in patients with chronic hepatitis C than hepatitis B (74% vs. 41%, $P = 0.015$). These results indicate that cryoglobulinemia and low CH_{50} levels would be markers of lymphoproliferative disorders characteristic of the patients with chronic hepatitis C.

Oligoclonality of the Immunoglobulin Heavy Chain (Ig-V_H) Gene in Patients With HCV Infection

Fingerprinting assay of the Ig-V_H gene was performed on B cells recovered from patients infected with HCV. Ladders of PCR products from B cells of healthy controls did not produce strong bands (Fig. 3A). In contrast, the ladders of some patients with HCV infection contained a monoclonal band indicative of the oligoclonality

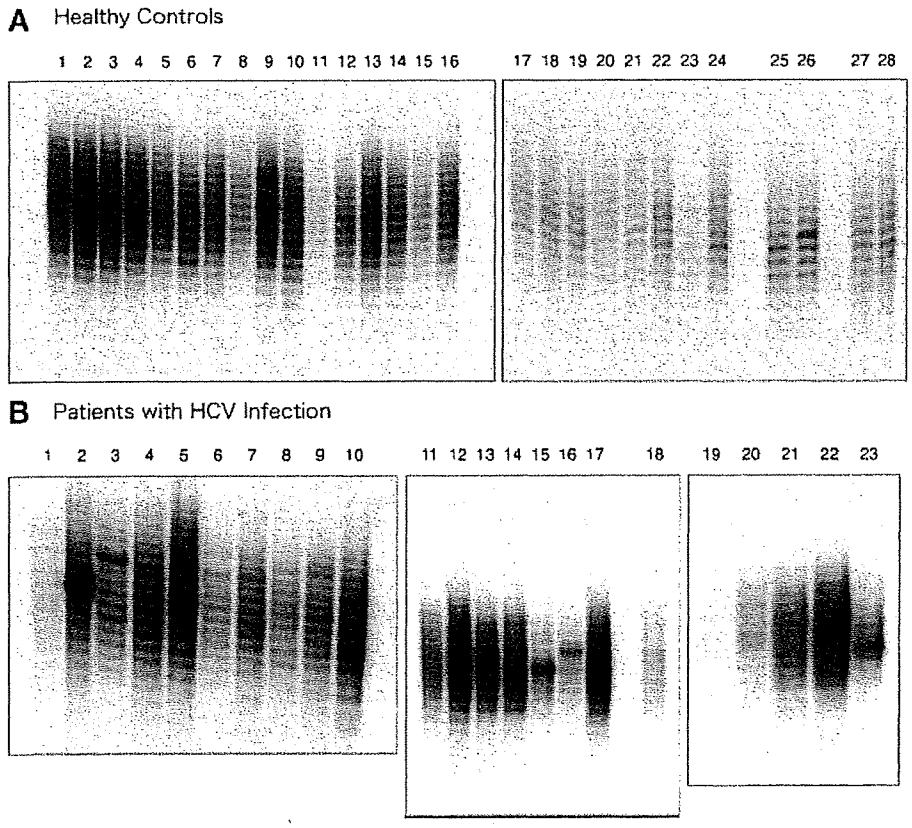

Fig. 3. Ig fingerprinting assay of (**A**) PBMCs from 28 healthy subjects and (**B**) B cells from 23 patients infected with HCV.

(Fig. 3B). The oligoclonality was observed not only in the patient with non-Hodgkin's lymphoma (lane 2 in Fig. 3B), but also in those without non-Hodgkin's lymphoma (lanes 3, 15, 16, and 23 in Fig. 3B). Overall, it was detected in B cells from 8 (11%) of the 75 patients infected with HCV, including one each with non-Hodgkin's lymphoma and hepatocellular carcinoma and six with chronic hepatitis. Six of these eight patients had B cells carrying HCV RNA, and seven possessed at least one of cryoglobulinemia (detected in three patients), high-titered RF (four patients) and hypocomplementemia (four patients). Six patients were infected with HCV group 1 and the remaining two with HCV group 2. When the same assay was undertaken on PBMCs from 32 patients with chronic hepatitis B, the oligoclonality was not observed in any of the patients (Table II).

Amino acid sequences of some clones, obtained from a patient whose Ig-V_H gene showed the oligoclonality (lane 3 in Fig. 3B), were similar (Fig. 4B). In contrast, clones obtained from a healthy subject without strong bands in Ig-V_H fingerprinting assay (lane 8 in Fig. 3A) were not similar in the sequence (Fig. 4A). These results suggest that a clonal expansion of B cells would occur in the peripheral blood of patients infected with HCV.

Factors Associated With Lymphoproliferative Disorders

Univariate analysis was performed to determine factors predisposing to lymphoproliferative disorders, including cryoglobulinemia, high-titered RF, hypocomplementemia and the B-cell clonality (Table III). Low ALT levels and the presence of HCV RNA in B cells were associated significantly with lymphoproliferative disorders. Among them, HCV RNA in B cells was the only predictive factor by multivariate analysis (odds ratio 1.98 [95% confidence interval 1.36–7.24], $P = 0.027$).

DISCUSSION

It has been demonstrated epidemiologically that HCV induces a number of extrahepatic manifestations [Cacoub et al., 1999; Zignego and Brechot, 1999], of which lymphoproliferative disorders is related most closely to HCV infection [Zignego et al., 2007]. Accordingly, it has been accepted that chronic infection with HCV can lead to the clonal expansion of B cells and that the sustained proliferation of B cells would promote the occurrence of genetic mutations. Zignego et al. [2000] have observed frequently t(14;18) translocation and overexpression of bcl-2 in lymphoid cells from patients with lymphoproliferative disorders in association with

(A) IgV$_H$ Gene Clones from a Healthy Control

```
C3   GDGDRGPLAP DVHTVVGTNA AILQRTVIHG RVVGSQATQL HVGCARGCVC GHRDSALELL CIVCVTIVSA DPSHPLKPLP RGLPHPADSI AGKGVTRSPA GDLH
C1   -------F-- ---V--VVGV -ATTT-T-TS T-IHGCVLGF QAVHLQIGDA PGII-*DGES AFHRRGVFCC PTISFASNET YP-QPLPW-L -NPAHSIITK -ESR
C2   ----QSS--- G-EP-YPLRV VPPHLSRTVI HGRVRSGHRA RLQGELALGR VY*YGDSTLE GG-V-GAPTV IYIPNPLQSL PWGLLDPAPV VTTDGATRDS EGEG
C13  ----Q-S--- V-KV-WASCC YSSTVIHGCV LGFQAVHL-I GDAPGIIS*D -ESA-HRRGV FCCPTISFAS NETY--Q--- WS-AD--H-- IT--EP--CT -ESQ
C10  -------P-- ---I--RPEP VNPTPDFCTL IEG-ALSREA AHL*IQ-VPA VVSGDGESA- HRV-IECCST I-TNKGD-LQ -LPWSLTYPM HGIIFKCESR -CTG
C7   ----Q-S--- V-NRGGK*FQ PGTVIHGRVL GSQAVHLQIQ *-LGVVSGDG ESALHRVCVV --TTTT-NV* N-LQ--PWSL ADPA-VVVTE GESRGCTGES QGPS
C12  R---HC---- -IKSIR*LPE P*KAG-RTVI HSRVLVSQAV -LLIQ*VLGI VSGDGESALH RVCV-YGTTT TTNV*NPLQ- LPWSLADPVH -IATEGE-RG CTGE
C16  -------F-- ---V-*ASQN IVSS----RS --L----VH- QIQ*VL-VVS -DGE---H*V -V-Y--TTTT NG*D--Q--- WS-ADPVHA- -TE-ESRGCT -ESC
C14  ---VQ----- V-K-*PQST- -AARSNRTVI HGRVLGSQAV -LQIQ-VLGI IEG-GEPA-H GVCI-CATTT AINN*DPLQ- LPWSLADPAH GIAAKGESRG CTGE
C5   ----Q-S--- QP*ISRTVIH GCVV-SQPAQ LHV-CADRRV PGHGDPALKL LCIVCATTVR VDPSHPLKPL SRGLSHPVHI VAGEDVSRSL ---DLH
C4   ----E-XPG- SSQSKQLALX HXFSHSNTRP CPRLSGCSFA DTVSSWR-XW RW*IGPSQSX RSL-NYYYX* CMKPTPA-SL EPXGSSSCS- H*R*IQRLHR R-X-Q
C6   PPAAPKGEFA AAKFNSPYSE SYYNSLAVVL QRRDWENPGV TQLNRLAAHP PFASWRNSEE ARTDRPSQQL RSLYVRQFKV YTYKRESRYR LFVDVQSDII DTPGR
C8   MQPPNISLIS LAVKRIICYI **KVNTFTKT RRFSN*TF*S T*LYMAYRTY ALNIRLYAVP VNGMPHL*TK EFSPDEMGVF KYPPSQGLWQ EYGLLL*CQT APAAP
C11  SRATVISAKT AR*STTCTYS R*RSPERARA IGISTT*CRR CPENPSTARF PFCKLLQSSI ILCRNQQGQA EDCIAHTCAS VDDTRMARTF STLRQLVVAV ICPAS
C9   -W-G*LQDKT *SRKRKYL*S KHFT*LSFCI YKL-IGREEX MGRGG**GS* LEVGSQTGIT LKT*RSRNRG KQTSARWQNR LF*CFSARLH QLH
C15  ETVTRAPWPQ *SND--APRS KGCLAQ*YTA VS-AVTELSF R-NWFLDVST DMVTRL-REG L*LVLAL**I PIHSSPFPGA AGSSSSSNY* WSHQGQC-RG T-SPK
```

(B) IgV$_H$ Gene Clones from a Patient Infected with HCV

```
HC17 GDGDRGPLAP DVHTVVGTTT IYGCRTVIHG RVLGSQATQL HVGCAYGHVF GNGDSFLEVL CVVCVITAND DPSYPLKALS TSLSHPLHSR TSKGEYRSLA GDLH
HC5                            S                                                                    S
HC10 ---------- ---------- ---------- ---------- -----C---- ---------- ---------- V--------- ---------- -----S-G-- ----
HC16 ---V------ --------A- ----H----- ---------- -----C---- ---------- ---------- --------M- ---------- -----S---- ----
HC8  ---------- ---------- -H-------- ---------- -----C---- -----L--L- -----T---- ---------- ---------- AT---S---- ----
HC15 ---------- -------S-- -*G------- -------A-- -----C---I -------L-- ------T--- ---------- ---------- G----S---- ----
HC2  ---------- ---------- -*----I-R- -------A-- ----PC---- T-G-----L- -----A---- G----P---- --PP------ G----S---- ----
HC24 ----Q-S--- V-X-FLNSTV ATI*C----- ---R---A-- ----VCRC-I G----A-L-- -I---TIVSA ---H--*P-- RG---SADTT AG--VT---- ----
HC7  ----Q-S--- VPYLQAANSR TVIHSRVLSS QAVHL-IQRV LGIVSGDGES ALHGVCVVFI TSII*YNCHP LQPLAWSLAD PVH-IASEGE SRGCTGESQG PPRL
HC26 R---HC---- -IKSSVAP-S PQLCSRTVIH SRVLVSQAVH LQIQ*GLGIV SGDGESALHR VC-VYG-TTT TNV*NPLQAL PWSLADPVHI ATEGESRGCG ESQ-
HC18 ----Q-SWPQ *SX*SNQPLL Y*SLAQ*YTA VSAAVTELSF RENWFLDVST DMVSRL-REG L*LVLPL**I YPIHSSPFPG GCRVQLQ**L LMEPPETVQV RGRV
HC4  --S-Q-AT-- ETEVP-VAA- TTTPPISRTV IYSRVCGAH* AQPQGELV-G RVYVCSDSS- EGRV-EG-PT SKHTPNPLQP LLWGLADPGP VVVTTDDGVT KDSA
HC12 ---------- ------VVRI RDNSFNHMPS T-IYGRVLGS QAVHLQIQRV LGIVSGDGKP ALHG-SVVCV TTTSIN**DP LQPLPWSPAD PVHGIRAKGG SRGY
HC13 ---------- -----LPNRS H-DGGISRTV IHGRVLGSQA VHLQIL*VLG VVSGDGEPA- HGVCVVCTTT ATTNG*DPLQ PLPRSLADTT HGIVPKGESR _CTG
HC6  ----Q-S--- V-XGGVI--I -IVPTPKIFH T-IYGRVLGS QAVHLQIQRV LGI-PGDGES ALHG-CIVPI TSIIPYNCHP LQPLAWSLAD PVHAIATEGE SRRC
HC25 ----Q-S--- V-XVPRI-PR TLPISRTVIH SRVLGSQAVH LQIQRVLGIV SGDGEWALHG VCIVFITSII *YNCHPLQPL AWSLADPVHA IATEGESRGC TGES
HC22 --S-Q-AT-- VTEVPPI-SR C-CRTVIHSR VLDSHAVHLQ IQRVLELSLE MVNRPFA-SA *YLSLPSFHI TATHSSPSPG AWRTQCMP*L PKVDPDAAQD SLRD
HC19 ----Q-S--L G-EPAGS-LF RTVIHSRVL- SQVVHLQI-R VL-IVS-DGE SALHGVCI-F ITSIIPYNCH PLQPLAWS-A DPVHAIATQG E-RRFTGESQ _PPR
HC9  ----Q-S--- VPYLQAANSR TVIHSR-LSS QAVHL-IQRV LGIVSGDGES ALHGVCVVFI TSII*YNCHP LQPLAWSLAD PVH-IASEGE SRGCTGESQG PPRL
HC21    X X PQ    X  VVPP HTIVEI*PSH SNRQPLSSG  RHVHLHI AC YWSRLXXW*I GPSRSLHSSS YPHLLI*LPP  PAPCLEPGG HSACQNX*SV NAMR
HC2  ----Q-S--- VPQRNCSHLY PS-TVTHCRV LGSQAVH*-I Q*VLGVVSGD -RSALHRVHI VPLTSILLYV GPPLQPLPWP ADPAH-IATR GRSRGCTGRS QGPD
HC23 ----Q-S--- V-XMCSSYHH YHRISRTVIH GRALGSQAVH LHIQ*VLG-V SGDGESALHR VHIVFL-SIL LYVGHPLQPL PWSLADPAHP IATKGESRGC TGES
HC1  ---------- ------VVKV VQLPEPFLVS RTVIHSRVLG SQAVHLQIQR VLGVVSGDGE SALH*VSVVY VTTTTTNG*D PLQPL-WSLA DPVHAIATEG ESRG
HC20 R---HC---- -IKSS-AP-S PQLCSRTVIH SRVLVSQAVH LQIQ*GLGIV SGDGESALHR VCVVYG-TTT TNV*NPLQAL PWSLADPVHA IATEGESRGC TGES
HC14 ----Q-S--- V-NKAT---- -SPRTVIRSR VLGSQAVILE IQ*VLGVVSG DAESALHIVC VIYACTLLLM YETIISSPPG AWRTQFIS*L LKVHIQRIRR VSGT
HC11 ----Q-S--- V-NCTGWSFR TVI*GRVVDS ETVHL*IYSV LGIVSGDGE- ALHGVCVLCG ITMTNMCDPL QSPPWSLADP -HVRGTKGES RSCTGESQGP PG-Y
```

Fig. 4. Aligned amino acid sequences of (A) the 16 Ig-V_H gene clones from a healthy control (lane 8 in Fig. 3A) and (B) the 26 clones from a patient infected with HCV (lane 3 in Fig. 3B). Dashes indicate the sequence identity. Three clones from the patient with more than 95% homology are boxed.

TABLE III. Baseline Characteristics of the Patients With or Without Lymphoproliferative Disorders

Features[a]	Lymphoproliferative disorders		Differences *P* value
	With (n = 57)	Without (n = 17)	
Age (years)	58.3 ± 14.5	55.2 ± 12.4	0.430
Men	35 (61%)	11 (65%)	0.805
Cirrhosis	15 (28%)	2 (12%)	0.177
Hepatocellular carcinoma	12 (22%)	2 (12%)	0.345
ALT (IU/L [5–25][b])	80 ± 82	170 ± 232	0.025
Platelets ($\times 10^4$/mm^3 [15–40][b])	17.6 ± 6.5	16.2 ± 6.4	0.430
HCV serogroup 1	47 (82%)	11 (65%)	0.119
HCV RNA in serum (log/ml)[c]	7.0 ± 1.4	6.8 ± 1.7	0.730
Serum HCV RNA > 5.0 log/ml	50 (88%)	14 (82%)	0.570
HCV RNA in B cells[d]	41 (72%)	6 (35%)	0.006
HCV RNA in B cells (log copies)[c]	4.5 ± 3.2	2.5 ± 3.4	0.036

ALT, alanine aminotranspherase; NS, not significant.
[a]Data are no (%) or the mean ± SD.
[b]Normal interquartile range.
[c]Determined in 100 ng of RNA extracted from cells.
[d]Determined number and percentage of patients with HCV RNA positive in B cells.

HCV infection. Either or both of the association of HCV-E2 protein with CD81 and the infection of B cells with HCV are proposed to accelerate the clonality of B cells [Matsuura et al., 2001].

In the 75 patients with chronic hepatitis C, the frequency of B cells harboring HCV RNA, as well as HCV RNA titers in B cells, was 10-fold higher than those of the other lymphoid cells including $CD4^+$, $CD8^+$ T cells. The replication of HCV in B cells was demonstrated in some patients with high titers of serum HCV RNA by the detection of negative-strand HCV RNA species; they represent viral replication intermediates. Combined, these results strongly suggest that HCV has a tropism for B cells.

On the basis of B-cell tropism, HCV isolates might be classified into at least three subgroups. One subgroup is merely associated with the surface receptors of B cells, but does not replicate efficiently in these cells. The results indicate that most HCV isolates belong to this group. Such an association might induce signaling toward a prolonged cell survival. B cells might express unknown receptors for HCV at levels higher than the other lymphoid cells. In support of this view, the negative-strand HCV RNA is barely detected in PBMCs from patients with hepatitis C, although positive strands are found in these cells [Lanford et al., 1995]. CD81, which is proposed as one of HCV receptors, is expressed on B cells much more densely than on hepatocytes [Machida et al., 2005]. There would be another subgroup of HCV capable of infecting B cells and replicating efficiently in them. Such B-cell tropic HCV, however, was identified in only four (5%) patients in this study. Nonetheless, infection with HCV may trigger somatic mutations in B cells, for example, bcl-6, p53, and β-catenin, leading to their clonal expansion [Machida et al., 2004]. A third subgroup of HCV would neither infect nor adhere to B cells.

It needs to be pointed out that methods used to detect HCV infection in extrahepatic cells have not combined high levels of both sensitivity and specificity, so far. Therefore, the possibility remains for the replication of HCV in some patients with chronic hepatitis C who did not have negative-strand HCV RNA in B cells in the present study; the frequency of false-negative results could not be determined in them. More sensitive and specific assay systems are required for estimating the actual frequency of HCV replication in B cells in patients with chronic hepatitis C with or without non-Hodgkin's lymphoma.

The association of HCV was less frequent in T cells than in the cell fraction without markers for B or T in the present study. The non-B, non-T cell fraction contains dendritic cells, macrophages and other lymphoid cells that were not $CD4^+$, $CD8^+$, or $CD19^+$. Dendritic cells have been demonstrated to interact with HCV-like particles in vitro [Barth et al., 2005], and are infected with HCV in vivo [Kanto et al., 2004]. Radkowski et al. [2005] reported the persistence of HCV in macrophages, even after it has been eliminated by interferon therapy. It is possible that HCV RNA might be associated with or infect dendritic cells and/or macrophages in non-B, non-T cell fractions, in replication levels lower than those in B cells.

The correlation between HCV infection and cryoglobulinemia is established [Agnello et al., 1992; Agnello, 1995]. RF was detected in high levels in sera from patients with not only chronic hepatitis C but also chronic hepatitis B (Table II). Recently, it was reported that the patients with chronic HBV infection are nearly three-times more likely to develop non-Hodgkin's lymphoma than controls [Ulcickas Yood et al., 2007]. As HCV infection, therefore, HBV infection may lead to lymphoproliferative disorders. The frequency of low CH_{50} levels was higher, although low C4 levels were detected only 2% in patients with chronic hepatitis C (Table II). These results stand at variance with those in a French study [Dumestre-Perard et al., 2002], which has shown low levels of both C4 and CH_{50} among patients infected with HCV. In this study, no patients with chronic hepatitis C had any cryoglobulinemia-related clinical syndrome, such as skin rashes, membranoproliferative glomerulonephritis and neuritis.

Hence, low C4, rather than CH_{50}, levels might be pathogenic and induce immune reactions in patients with chronic hepatitis C.

A correlation was sought for between infection and/or association of B cells with HCV and the occurrence of lymphoproliferative disorders. HCV RNA in B cells was an independent factor correlated with at least one of markers for lymphoproliferative disorders in multivariate analysis. Therefore, infection and/or association of B cells with HCV may lead to lymphoproliferative disorders, although the mechanism remains unknown. It is possible that infection of B cells with HCV would induce somatic mutations or over-expression of anti-apoptotic genes toward a prolonged survival of activated B cells. Or else, mere interaction between envelope proteins of HCV and signaling receptors on the cell surface, which regulate the survival of B cells, can be involved in the genesis of lymphoproliferative disorders.

The clonal expansion of B cells was reported to occur in 26% of Italian patients [Pozzato et al., 1999], while it has not been observed in any Japanese patient investigated so far. The detection of B-cell clonality in 11% of Japanese patients in this study, however, would point to a possibility for HCV-induced lymphoproliferation not dependent on ethnicity. Several studies have focused on important roles of sustained antigenic stimulation, analogous to lymphomagenesis due to infection with *H. pylori*, in a possible relevance to the extra-nodal marginal-zone B-cell lymphoma arising in lymphoid tissues on mucosae (MALT lymphoma) [Ivanovski et al., 1998; De Re et al., 2000; Sansonno et al., 2004]. Further studies are necessary to clarify molecular mechanisms for the generation of lymphoproliferative disorders and the correlation between malignant lymphoma and lymphoproliferative disorders.

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