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Prominent proliferative response of peripheral blood mononuclear cells to a recombinant non-structural (NS3) protein of hepatitis C virus in patients with chronic hepatitis C

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SUMMARY

The proliferative response of peripheral blood mononuclear cells (PBMC) to a recombinant non-structural (NS3) protein of hepatitis C virus (HCV) was studied in 41 patients with chronic hepatitis C. Of them, 28 had chronic persistent hepatitis (CPH) and 13 chronic active hepatitis (CAH). The positive proliferation rate of PBMC to the recombinant NS3 protein, T9Ag, was 66% in the 41 patients (77% in CAH *versus* 61% in CPH; $P > 0{\cdot}05$) when stimulation index (SI) = 4 was set as the cut-off value. However, mean SI of CAH patients was significantly higher than that of CPH patients ($8{\cdot}3 \pm 5{\cdot}2$ *versus* $5{\cdot}1 \pm 3{\cdot}6$; $P < 0{\cdot}05$). Six other chronic hepatitis patients who were repeatedly negative for anti-HCV antibody but positive for serum HCV RNA also had an SI of $\geqslant 4{\cdot}0$. The frequency of cellular immune response to the T9Ag is among the highest results obtained by using HCV antigens tested so far. Our studies thus indicate that NS3 is an immunologically important region of HCV for T cells. Moreover, the proliferative response to T9Ag may help to establish hepatitis C etiology in chronic hepatitis patients who are seronegative with currently available anti-HCV assays.

Keywords immune response chronic hepatitis C non-structural protein hepatitis C virus peripheral blood mononuclear cells

INTRODUCTION

Since the advent of serological assays for hepatitis C virus (HCV), HCV has been shown to be the major etiologic agent for post-transfusion and sporadic non-A, non-B hepatitis all over the world [1–3]. At least half of the infections become chronic, and those infected suffer a protracted clinical course [4,5] with a significant proportion of them progressing to end-stage liver disease and liver cancer [6]. Mechanisms for persistent infection as well as hepatocyte necrosis remain unknown. Antibodies directed against several HCV-encoded proteins have been extensively characterized and used in serological diagnosis, but the antibody titres do not correlate with disease course [7,8]. Cellular immunity, on the other hand, has barely been investigated. Although HCV-specific cytotoxic T lymphocytes recognizing epitopes in the core and envelope proteins of HCV have been cloned from liver tissues of patients with chronic hepatitis C [9], and cytotoxic T lymphocytes have also been detected in hepatic infiltrates [10,11], the roles of these lymphocytes remain unclear. As they may be involved in the mechanisms of the chronicity and immunopathogenesis of HCV infection, more studies are needed.

T cell proliferative responses to hepatitis virus-encoded proteins have been shown in patients with chronic hepatitis induced either by hepatitis B virus (HBV) [12,13] or by HCV [14–16]. Acute exacerbations of chronic type B hepatitis were shown to be correlated with increased T cell responses to hepatitis B core antigen (HBcAg) as well as to hepatitis B e antigen (HBeAg), indicating that HBcAg- or HBeAg-specific $CD4^+$ T cells may be responsible for mediating hepatitis activity [12]. For hepatitis C, three recent studies [14–16] have employed recombinant HCV antigens, including structural and non-structural proteins, to stimulate *in vitro* peripheral blood mononuclear cells (PBMC) of patients with chronic hepatitis C. Among them, the proteins originating from NS3-4 (C-100) or core (C22) regions were documented to be more immunogenic in these patients. In patients with chronic HCV infection, we also confirmed that a prominent T cell proliferative response could be detected against a recombinant protein

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(T3Ag) which overlaps with C-100 [17]. However, only one study has ever tested the immunogenicity of protein exclusively from the NS3 region [16]. Since anti-NS3 is one of the most dominant humoral immune responses observed in patients with HCV infection [18,19] it would be interesting and important to know more about the cellular immune responses to NS3 protein.

In this study, we produced a recombinant protein, T9Ag, encompassing the major middle portion of the NS3 region, and used this protein to study the proliferative responses of PBMC from patients with chronic hepatitis C. We found that 66% (27/41) of patients had strong T cell responses to this antigen. We also detected a positive proliferative response to T9Ag in six chronic hepatitis patients who were seronegative for antibody to HCV (anti-HCV).

PATIENTS AND METHODS

Patients

Forty-one patients (29 male, 12 female; median age 47 years, range 24–68 years) with chronic hepatitis C were recruited into this study. The diagnosis of all patients was based on (i) strong seropositivity (optical density (OD) > 2·0) for antibody to HCV (anti-HCV), which was detected by a second-generation enzyme immunoassay (anti-HCV-II; Abbott Laboratories, North Chicago, IL); and (ii) histologic diagnosis after percutaneous needle biopsies. The histologic diagnosis was according to the criteria of an International Group [20]. Thirteen patients had chronic active hepatitis (CAH) and 28 chronic persistent hepatitis (CPH). All were seronegative for hepatitis B surface antigen (HBsAg) (Ausria; Abbott Labs) and HIV (HIV Enzygnost Anti-HIV Micro; Behringwerke AG, Marburg, Germany) and none had a history of alcohol or drug abuse.

Ten age-matched patients with chronic hepatitis B who were seronegative for anti-HCV served as controls. Six other chronic hepatitis patients who were repeatedly seronegative for anti-HCV were also recruited in the study.

Construction of T9 antigen (T9Ag) expression plasmid

The cDNA coding for T9 antigen (from aa 1188 to aa 1493) was amplified by polymerase chain reaction (PCR) from the cDNA template of the Taiwanese strain of HCV [21]. The sequence of the forward primer is 5′-TTGGATCCGTGGCTAAGGCGGTGGACTT-3′ and the sequence of the reverse primer is 5′-TTGGATCCCTATTACCTGCCTCGCCGCTGCGAGCG-3′. Underlinings indicate the *Bam*H1 site which was created at both ends of the PCR insert in order to facilitate cloning. The resulting DNA was cloned in frame into a pET3a expression vector [22] at the *Bam*H1 site to yield the expression plasmid pT9 (Fig. 1a).

SDS–PAGE and Western analyses of T9Ag expression in Escherichia coli

Plasmid pT9 was first transformed into *E. coli* BL21 (DE3). The transformants were grown overnight at 37°C in Luria broth (L broth) containing ampicillin (50 μg/ml). On the second day, the cultures were diluted 1:100 with L broth containing ampicillin. The cultures were grown to OD 600 = 0·5–1 unit. After addition of 0·4 mM isopropyl-β-D-thiogalactoside (IPTG) cells were incubated at 37°C for an additional 1 h and then harvested. Total lysate proteins from the cultures before induction and 1 h after induction were analysed by SDS–PAGE [23]. To further confirm the identity, Western blot analysis was performed. Proteins resolved in the SDS–PAGE were electrotransferred to a nitrocellulose filter [24]. The filter was first blocked with 5% non-fat milk dissolved in TBS buffer (0·05 M Tris–HCl, 0·9% NaCl, pH 7·6) at room temperature for 2 h, followed by incubation with chronic hepatitis C patient's serum (1:500 dilution) at room temperature for 2 h. After extensive washes six times with TBS–T (0·25% Tween-20 in TBS), a second antibody, goat anti-human IgG, conjugated with horseradish peroxidase (HRP), was added at a 1:10000 dilution and incubated at room temperature for another 2 h. The filter was again washed six times with TBS–T, and then developed with the ECL detection reagent (Amersham, Aylesbury, UK).

Purification of T9Ag

Following cell breakage and centrifugation, the insoluble T9Ag was first dissolved in 1:3 volume of the original lysate of 0·3% SDS in buffer A (50 mM Tris pH 7·5, 50 mM NaCl, 1 mM EDTA, and 0·15% β-mercaptoethanol). The residual insoluble debris

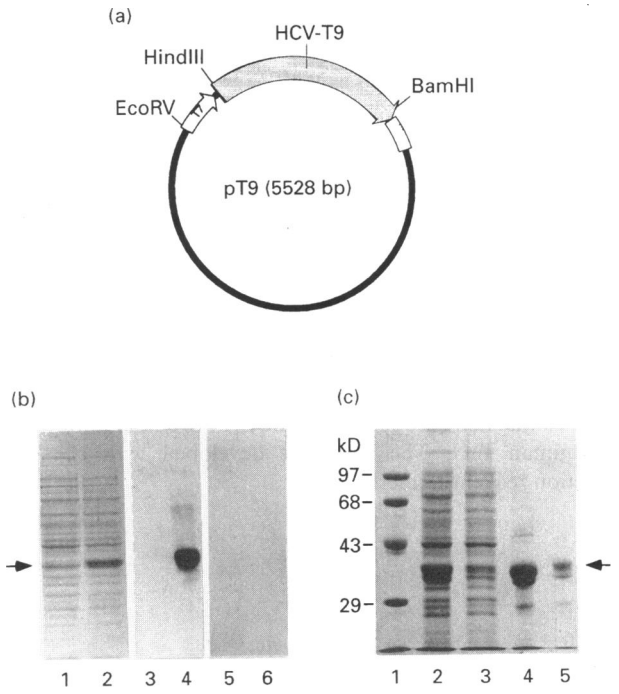

Fig. 1. Expression and purification of T9Ag. (a) Diagram of the T9Ag expression plasmid, pT9. The cDNA fragment codes for aa 1188–1493 of the hepatitis C virus (HCV) polyprotein. (b) Expression of T9Ag in *Escherichia coli*. Lanes 1, 2, SDS–PAGE analysis of total lysate proteins; lanes 3, 4, Western blot analysis of total lysate proteins with serum from a chronic hepatitis C patient; lanes 5, 6, Western blot analysis of serum from a healthy control. The odd number lanes are the lysate proteins from the culture before induction, whereas the even number lanes are the proteins from the culture after 1 h induction. Arrow indicates the position for T9 antigen. (c) Purification of T9Ag. Lane 1, molecular weight size markers (Bio-Rad Laboratories, Hercules, CA); lane 2, initial total lysate proteins; lane 3, proteins which remained in the supernatant after centrifugation of total lysate proteins; lane 4, proteins dissolved with 0·3% SDS from the inclusion body; lane 5, final purified products from the Sephacryl S-200 HR gel filtration chromatography. Arrow indicates the position for T9 antigen.

was removed by centrifugation, the solution was further concentrated with Amicom centriprep-10 to 1 : 10 volume of the original lysate, and then subjected to Sephacryl S-200 HR gel filtration chromatography (Pharmacia Biotechnology, Uppsala, Sweden) with 0·02% SDS/buffer A as eluent. Fractions containing the T9Ag were determined by SDS–PAGE analysis and further confirmed by Western blot analysis. Positive fractions were pooled and analysed for antigen purity and quantity.

Separation of PBMC

PBMC were separated from fresh heparinized blood by centrifugation over a Ficoll–Hypaque density gradient [25].

Proliferative assay

PBMC were cultured in 96-well flat-bottomed microculture plates at a density of 3×10^5 cells/well in complete medium containing RPMI 1640 medium, 5% fetal calf serum (FCS), L-glutamine and antibiotics. Cultures were incubated for 7 days at 37°C in humidified 5% CO_2 atmosphere in the presence of different final concentrations of T9Ag (0, 0·5, 1·0, 2·0, 4·0 and 8·0 μg/ml). Proliferative assays were performed in triplicate with 0·5 μCi/well ^{3}H-thymidine (^{3}H-TdR; specific activity 2 Ci/mmol; Amersham) added to each well 16 h before harvesting. Cells were harvested with a Skatron cell harvester (Lierbyen, Norway). The ^{3}H-TdR incorporation was then determined by a β-counter. The data were expressed as ct/min and the stimulation index (SI = mean ct/min of antigen-stimulated cultures/mean ct/min of control cultures). Lysate protein extracted from pET vector-transformed *E. coli* was used as control antigen.

Western blot analysis

To analyse anti-T9Ag antibodies in patients' sera, the purified T9Ag first resolved by 12·5% SDS–PAGE was transferred from the gel to a nitrocellulose filter [24]. The filter was reacted with patients' sera (1 : 500 dilution) and subsequently with an HRP-conjugated secondary antibody (1 : 10 000 dilution), goat anti-human IgG. The blot was developed with the ECL detection reagent (Amersham).

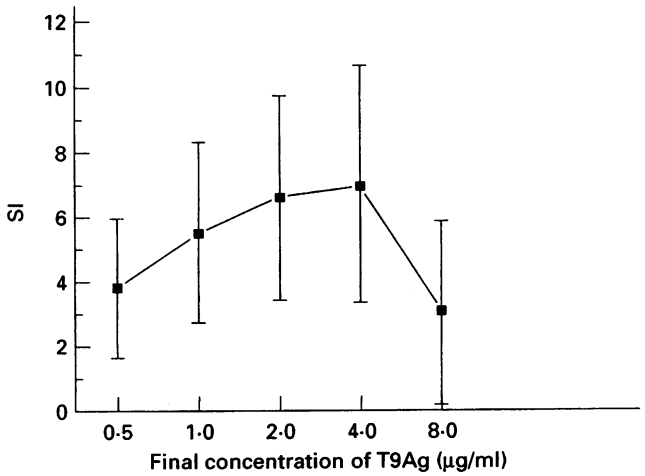

Fig. 2. Dose response for T9Ag stimulation of peripheral blood mononuclear cells from nine patients with chronic hepatitis C. Vertical bars represent mean ± s.d.

Statistical analysis

χ^2 test with Yates' correction and Mann–Whitney *U*-test were used as indicated. $P < 0.05$ was considered statistically significant.

Table 1. Proliferative response of peripheral blood mononuclear cells (PBMC) to a recombinant protein (T9Ag) of hepatitis C virus (HCV) in patients with chronic hepatitis C

Patient no.		ALT(U/*l*)*	Stimulation index of PBMC	(ct/min)
CAH†	1	200	10·7	(30 305)
(*n* = 13)	2	12	10·5	(30 638)
	3	100	9·9	(30 441)
	4	85	1·9	(4101)
	5	100	16·5	(34 308)
	6	72	0·7	(1220)
	7	80	8·5	(16 224)
	8	25	16·5	(18 782)
	9	25	8·7	(14 982)
	10	277	12·2	(26 383)
	11	32	2·4	(7972)
	12	42	4·3	(9565)
	13	200	4·9	(6858)
Mean± s.d.		96·2±81·4‡‡	8·3±5·2‡	(17 829±11 494)
CPH†	1	54	4	(4037)
(*n* = 28)	2	16	5	(21 288)
	3	60	3·4	(4224)
	4	70	4·8	(4643)
	5	16	6·1	(19 049)
	6	33	10·4	(9234)
	7	17	7·9	(22 620)
	8	37	4·2	(4428)
	9	100	0·3	(890)
	10	42	0·7	(1213)
	11	65	12·7	(36 613)
	12	40	9·9	(16 852)
	13	22	6·8	(10 253)
	14	16	5·9	(11 353)
	15	16	0·2	(666)
	16	74	11·5	(26 888)
	17	50	1·2	(2343)
	18	102	1·8	(2381)
	19	112	7·9	(21 788)
	20	67	9·4	(13 651)
	21	300	1·6	(2616)
	22	14	2·8	(6745)
	23	60	7·6	(12 113)
	24	39	4·2	(8780)
	25	44	6·3	(5001)
	26	70	2·1	(5126)
	27	18	0·7	(1217)
	28	82	3·4	(5129)
Mean ± s.d.		58·4 ± 55·2‡ ‡	5·1 ± 3·6‡	(10 041 ± 9183)

* Serum ALT levels were measured on the same day.

† CAH, Chronic active hepatitis; CPH, chronic persistent hepatitis; ALT, alanine aminotransferase.

‡ $P < 0.05$; ‡ ‡ $P > 0.1$, Mann–Whitney *U*-test.

Table 2. Stimulation index (SI) of chronic hepatitis C patients under T9Ag stimulation

	SI ⩾ 4, %	Mean of SI (± s.d.)	Range	SI ⩾ 8·5, %
CPH ($n = 28$)	61 (17/28)	5·1 ± 3·6*,**	0·2–12·7	18 (5/28)†
CAH ($n = 13$)	77 (10/13)	8·3 ± 5·2*,***	0·7–16·5	62 (8/13)†
Total ($n = 41$)	66 (27/41)	6·1 ± 4·4****	0·2–16·5	32 (13/41)
Controls ($n = 10$)	0	1·6 ± 0·6**,***,****	0·2–3·4	0

* $P < 0·05$; ** $P < 0·001$; *** $P < 0·001$; **** $P < 0·001$ (Mann–Whitney U-test).
† $P < 0·05$, $\chi^2 = 5·94$ (χ^2 test with Yates' correction).

RESULTS

Expression and purification of T9Ag

The cDNA coding for T9Ag ranging from aa 1188 to aa 1493 was cloned in a pET3a expression vector and the resulting plasmid transformed with *E. coli* BL21 (DE3). A protein of 38 kD in size was expressed after induction with IPTG; it was recognized by the serum from chronic hepatitis C patients but not by the serum from healthy control (Fig. 1b). After breakage and centrifugation of the cells, T9Ag was present mainly in inclusion bodies which were solubilized with 0·3% SDS. Subsequent Sephacryl S-200 HR gel filtration yielded a partially purified T9Ag. The purity of the antigen was around 65% as determined by densitometry (Fig. 1c). This protein was then used for the following proliferative assays.

Determination of dose-dependent curve

The dose-dependent curve of T9Ag stimulation shown in Fig. 2 was determined with the PBMC from nine patients with chronic hepatitis C. The optimal concentration of T9Ag for stimulation was 4·0 μg/ml, with a mean SI of 7·0. This concentration was therefore used for the following experiments, except where specifically indicated.

Stimulation index in subjects free of HCV infection

Of the 10 patients with chronic hepatitis B, nine had an SI < 2·0 and one had an SI of 3·4. HCV genome was not detected by a reverse-transcription PCR (RT-PCR) assay in the serum of the only patient whose SI > 2·0. Therefore, we defined a positive proliferative response as that with an SI ⩾ 4·0 when the PBMC were stimulated with various concentrations of T9Ag.

Proliferative response of PBMC from patients with chronic hepatitis C

Table 1 depicts the proliferative responses of PBMC to T9Ag in individual patients with CPH and CAH. It was noted that 66% (27/41) of the PBMC from patients with chronic hepatitis C had a positive proliferative response when stimulated with T9Ag (Table 2). In contrast, no patient responded to protein lysate extracted from the pET vector-transformed *E. coli* (data not shown). The positive rate of proliferative response to T9Ag of the PBMC from CAH patients was not significantly different from that of the CPH patients (77% *versus* 61%, $P > 0·05$, Table 2). However, the mean SI of CAH patients was significantly higher than that of CPH patients (8·3 ± 5·2 *versus* 5·1 ± 3·6, $P < 0·05$; Mann–Whitney U-test, Table 2).

Correlation of SI with serum alanine aminotransferase levels

In one CAH patient, the proliferative assay of his PBMC was performed four times during the follow-up course. The SI and ALT levels determined each time were as follows: 9·9 *versus* 100; 4·3 *versus* 42; 9·9 *versus* 32; and 4·9 *versus* 25, respectively, indicating that there was no correlation between the SI values and ALT levels in this patient. In addition, no correlation between SI values and ALT levels was noted either in the 41 patients with chronic hepatitis C.

Table 3. Clinical and laboratory data of six chronic hepatitis patients who were seronegative for anti-hepatitis C virus (HCV) antibody

Patient no.	Sex	Age (years)	HBsAg	Anti-HCV*	SI to T9Ag	Anti-T9Ag antibody†	HCV RNA
1	F	48	–	–	4·7	+	+
2	M	39	–	–	4·1	+	+
3	M	50	+‡	–	16·5	++	+
4	M	21	–	–	9·6	++	+
5	M	52	–	–	7·6	++	+
6	F	55	–	–	4·9	+++	+

* Determined by using a second-generation enzyme immunoassay.
† Determined with Western blot analysis (+ ~ +++ indicates degree of reactivity).
‡ This patient was at first recruited as a negative control, but positive proliferative response was noted during the study.
HBsAg, hepatitis B surface antigen.

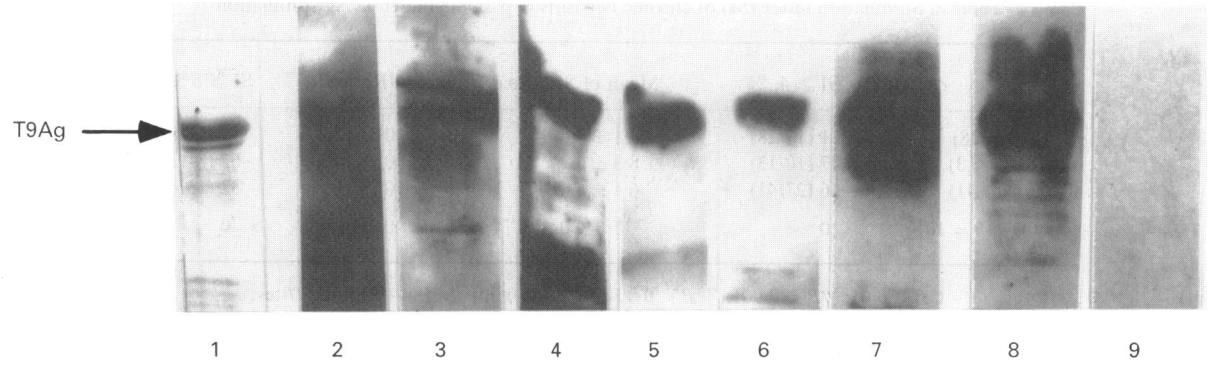

Fig. 3. Immunoblot analysis of the sera from six chronic hepatitis patients seronegative for anti-hepatitis C virus (HCV). The sera were tested for the sero-reactivities to the purified T9Ag. Lane 1, purified T9Ag resolved on a 12·5% SDS–PAGE; lanes 2–7, sera from the six chronic hepatitis patients seronegative for anti-HCV; lanes 8, 9, serum from an anti-HCV$^+$ patient and a healthy subject, used as positive and negative control, respectively.

Proliferative response of PBMC from chronic hepatitis patients seronegative for anti-HCV

In our study, we encountered six other chronic hepatitis patients who were repeatedly seronegative for anti-HCV by using a second-generation enzyme immunoassay (anti-HCV-II; Abbott Labs). Five of them were also seronegative for HBsAg. However, all of them had a positive proliferative response to T9Ag (Table 3). We detected HCV genomes in the sera of all these six patients by a RT-PCR assay [26], indicating that they were indeed infected with HCV. To confirm this notion further, we performed immunoblot analysis by using the purified T9Ag, and the sera from all six patients reacted with T9Ag (Fig. 3). The results are thus consistent with our T cell proliferative assay, and indicate that these sera were indeed devoid of detection by second-generation anti-HCV assays.

DISCUSSION

The T9Ag is a protein encoded exclusively by the NS3 gene of HCV and represents the middle part of the NS3 protein. Only Schupper and co-workers [16] have so far used C33c protein, encoded exclusively by the NS3 gene of HCV, in the proliferative assay. However, they determined percentages of S-phase cells after PBMC were stimulated *in vitro*, rather than assayed for ^{3}H-TdR incorporation. In our study, PBMC from 27 of 41 (66%) patients with chronic hepatitis C had an SI $\geqslant$ 4 under the stimulation of T9Ag. In addition, the extent of proliferation of PBMC from CAH patients in response to T9Ag was significantly higher than that of PBMC from CPH patients.

In a previous study, the positive proliferative rate of PBMC from patients with chronic hepatitis C to another HCV recombinant protein, T3Ag (which is encoded from the junction of NS3 and NS4 regions (aa 1454–1648) and overlaps with C-100), was 32% when the cut-off value of SI was set at 4·0 [17]. The results are similar to those reported in Italian patients, with a response frequency to C-100 protein of 39% [14] and 24% [15], respectively. In the present study, we also used T3Ag to stimulate the PBMC of chronic hepatitis C patients simultaneously, and obtained results (data not shown) similar to our previous study [15]. Therefore, the positive proliferation rate (SI $\geqslant$ 4) of PBMC from patients with chronic hepatitis C to T9Ag assayed with ^{3}H-TdR incorporation is the highest among all the HCV recombinant proteins tested so far. These data may also explain the previous observations that the positive rate of anti-C33c is generally higher than that of anti-C100-3 in patients with HCV infection [18,19], and that anti-C33c usually appears earlier in the course of acute hepatitis C [19].

Although we did not analyse the types of cells responsive to the T9Ag *in vitro* in every patient, the phenotype of the blast cells present in the PBMC after 7-day culture was studied in one patient. More than 90% of the blast cells were CD3$^+$CD4$^+$. Through limiting dilution, several CD4$^+$ T cell lines were obtained from this patient by stimulating his PBMC with T9Ag. Further characterization of these T cell clones is now in progress. Taken together, NS3 region of HCV is likely to contain an immunologically important protein for CD4$^+$ T cell and B cell responses [18,19]. Recently, we have completed the epitope mapping of NS3 protein for anti-HCV antibodies and identified four epitope regions within the protein which could be recognized by sera of patients with chronic hepatitis C (unpublished results).

The lack of correlation between T9Ag proliferative response and hepatitis activity in our study does not favour the pathogenesis mechanism, whereby cytotoxic T lymphocytes are induced by the T9Ag-specific helper T cells and thus cause chronic type C hepatitis. As a result, the biologic significance of the proliferative response against T9Ag may not lie in enhancing cytotoxic responses. The finding that proliferation of PBMC against T9Ag detected in patients with CAH was stronger than that in patients with CPH is intriguing. Whether and how the NS3 antigen is processed in hepatocytes and triggers periportal hepatocyte necrosis, a hallmark of CAH, is obscure. In the study of Botarelli *et al.*, T cell responses to core protein of HCV are associated with a benign course of HCV infection [14]. However, Koskinas and co-workers could not confirm this association [27]. This means that the biological role of cellular immune response to various parts of HCV protein remains unclear. Our finding is consistent with this notion, although we used T9Ag instead of core protein.

In our study, we have encountered six chronic hepatitis patients who were seronegative for anti-HCV, but responded definitely to the T9Ag. They were later proved to have HCV infection, as HCV RNA was detected in their sera. Immunoblot

analysis using the purified T9Ag also indicated that anti-T9 antibodies were present in the sera of all six patients. The results are therefore consistent with T cell proliferative assay, implying that T helper cells are needed to induce the formation of these specific antibodies. It is not clear why the anti-HCV kit could not detect antibodies in these sera, but immunoblot assay could, since the kit includes a C33c antigen which is very similar to our T9Ag. Since reduced SDS–PAGE was used in our study, it is possible that the linear epitopes recognized by sera of these patients were not well presented in the C33c preparation in the kit. Our finding indicates that the proliferative response of PBMC to T9Ag may help to define hepatitis C etiology in chronic hepatitis patients who are seronegative for anti-HCV checked with currently available assays. The fact that all these six anti-HCV-negative patients actually have antibodies against NS3 antigen calls for further improvement in detection of anti-HCV by using more immunoreactive peptides for the serologic assay kit.

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