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Establishment and Characterization of NS3 Protein-Specific T-Cell Clones from a Patient with Chronic Hepatitis C

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Abstract

Our previous study showed dominant proliferative response of peripheral mononuclear cells to hepatitis C virus (HCV) nonstructural (NS-3) (T9, from aa 1188 to 1493) in chronically infected patients. Six T9-specific T-cell clones derived in an HCV patient were established and studied for the antigen specificity and the ability of augmentation of in vitro antibody production. All these cloned T-cell lines responded exclusively to T9 antigen and could help autologous B cells in producing anti-T9 antibody in vitro. Cytokine mRNAs of these T cells was detected by polymerase chain reaction and predominant IL-2 and IFN-γ production was noted. In addition, further elucidation of T-cell antigenic determinant and MHC restriction suggested that these T-cell clones recognized at least two different T-cell antigenic determinants within the NS-3 region in an HLA DQ2-restricted manner. We believe characterization of HCV-specific T-cell responses, especially T-cell epitope mapping and cytokine production pattern, may shed light on further understanding the pathogenic mechanism and designing therapy for HCV infection.

Key Words
Hepatitis C virus
T-cell clones
Cytokines

Introduction

After cloning of the hepatitis C virus (HCV) genome [7], many studies on further characterization of clinical features of HCV have been completed. Using commercially available kits, HCV has been found to be responsible for about 60–90% of non-A, non-B posttransfusion hepatitis [6, 18]. Most importantly, hepatitis C infection is characterized by an unusually high frequency of chronicity after acute infection and about 20% of them will develop cirrhosis and hepatoma [1, 16].

The mechanisms of this chronicity, however, remain largely unknown. Lack of effective neutralizing antibody, escape mutation of cytotoxic T-cell (CTL) epitope and imbalance between T-helper cell cytokines have all been suggested to explain the persistent HCV infection [3, 4]. Although many studies concerning HCV epitope recognized by cytotoxic T cells have been reported [15, 26], it is still controversial about the existence of mutation epitope in HCV infection. It has been well documented that NS-3 region protein is the major B-cell epitopes during the infection. Interestingly, we found that antibody to a region within NS-3 protein (protein A, aa 1250–1334) was less in cirrhotic patients than in chronic hepatitis patients [14]. We have previously demonstrated that 66% of HCV patients had strong T-cell responses to the T9 antigen

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(part of the NS-3 protein, aa 1188–1493) [29]. Since imbalance between TH_1 and TH_2 has been suggested to play an important role in the defense mechanisms of several diseases such as human immunodeficiency virus (HIV) or leishmania infection [9, 10, 24]. It is speculated that the discrepancy in cytokine secretion plays a role in the disease severity and pathological process in liver disease [11].

In the present study, we further characterized T-cell antigenic determinants of NS-3 proteins and investigated the pattern of cytokine production of cloned T-cell lines. The information may shed light on understanding the immune response of HCV infection and provide help in diagnosis and even future vaccine design.

Materials and Methods

Patient and Cell Separation

A 50-year-old man was positive for antibody to HCV (Abbott HCV EIA 2.0; Abbott Laboratories, North Chicago, Ill., USA) and was diagnosed to have chronic active hepatitis. He was followed at our clinic. Liver biopsy revealed mild cirrhotic changes. To isolate peripheral blood mononuclear cells (PBMCs), heparinized blood samples were collected from venipuncture and isolated by using Ficoll-Hypaque (Pharmacia Diagnostics AB, Uppsala, Sweden). Cells at interface were removed carefully and washed twice before experiments.

Reagents and Culture Medium

Appropriate fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies such as anti-HLe-1 (CD45, clone 2D1), anti-Leu-M3 (CD14, clone MO-P9), anti-Leu-2a (CD8, clone SK1), anti-Leu-3a (CD4, clone SK3), anti-Leu-4 (CD3, clone SK7), anti-Leu-12 (CD19, clone 4G7), anti-Leu-18 (CD 45RA, clone L48), anti-TCR 1 (α/β, clone WT31) and anti-TCR 2 (γ/δ, clone B1.1) (Becton Dickinson, Mountain View, Calif., USA) were used for phenotypic analysis of T-cell clones. Most cells were cultured in the RPMI medium supplemented with 1 m*M* glutamine, 1 m*M* sodium pyruvate, 5×10^{-5} *M* 2-ME, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2% human-type AB serum (ABS). For the first 10–14 days of culture, T cells were cultured in serum-free medium (AIM-V; Gibco, Gaitherburg, Md., USA).

Purification of NS-3 Region Gene-Encoded Proteins

To purify the proteins used in defining T-cell antigenic determinants, plasmids containing pET-HCV NS-3 sequences were transformed into *Escherichia coli* BL21(DE3). Cells were grown overnight at 37°C in M9ZB broth containing ampicillin (50 μg/ml). On the second day, the cultures were diluted 1:20 with M9ZB broth containing 150 μg/ml ampicillin, 3 times the cell concentration of that normally used in order to increase the efficiency of induction. The cultures were grown to an OD_{600} = 0.5–0.7 unit. After adding 1 m*M* isopropyl-β-*D*-thiogalactoside (IPTG; Sigma Co., St. Louis, Mo., USA), cells were incubated at 37°C for an additional 2 h and then harvested by centrifugation. Cells were broken by sonication, inclusion bodies were obtained by centrifugation at 12,000 rpm for 15 min, and dissolved in binding buffer (5 m*M* imidazole, 500 m*M* NaCl, and 20 m*M* Tris-HCl pH 7.9) containing 1% SDS. The recombinant proteins were purified by nickel-affinity column (Gibco BRL Life Technologies, Inc., Grand Island, N.Y., USA). Each fraction was collected and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The positive fractions in 1 × PBS buffer containing 1% SDS were pooled and dialyzed at 4°C with SDS in concentration decreasing from 0.5 to 0.02%, finally in 0.01% SDS. The dialyzed antigens were concentrated to 0.5–1 mg/ml with Centriprep (Amicon, Inc., Beverly, Mass., USA). All recombinant proteins were at least 80% pure as judged on the basis of Coomassie blue staining and SDS-PAGE.

Establishment of T9-Specific T-Cell Clones

To establish the T9-specific T-cell lines, PBMCs were stimulated with 4 μg/ml T9 antigen in serum-free medium. Three days later, recombinant human IL-2 (with a concentration of 10 IU/ml) was added to the culture. Ten to 14 days later, the viable cells were harvested and plated in 96-well plates at a concentration of 10^4 cells/well, activated with 10^5 cells/well irradiated PBMCs (4,500 rad) or Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (EBV-LCL, 10,000 rad) as antigen-presenting cells (APCs) and 4 μg/ml T9 antigen. T cells were expanded and screened by proliferative response to T9 antigen. To further subclone the established T-cell lines, T cells were plated onto 96-well plates at a concentration of 0.3 or 1 cell/well. T cells were fed with APCs plus antigens in medium containing 10 IU/ml IL-2 every 2 weeks. For positive clones, the cells were expanded before further characterization.

T-Cell Proliferative Responses to Specific Antigens

To test the antigen specificity, 5×10^4 cloned T cells were stimulated with irradiated EBV-LCL (5×10^4 cells/well) or PBMCs (1×10^5 cells/well) with or without antigens in 96-well plates. Three antigens, the T9 antigen (2 μg/ml), HCV core antigen (1 μg/ml) and tetanus toxoid antigen were tested in proliferation assay. After incubation at 37°C for 5 days, each well was added 1 μCi ^{3}H-thymidine and incubated for another 16 h. Cells were harvested onto filter papers and radioactivity was determined with a β-counter (Parkard Instrument Co., Meriden, Conn., USA).

Microfluorometric (FMF) Analysis

The PBMCs obtained by Ficoll-Hypaque density centrifugation were used for phenotypic analysis by FMF. Aliquots of cells ($2.5–5 \times 10^5$ cells) were suspended in RPMI 1640 medium and incubated at 4°C for 30 min with predetermined optimal concentration of appropriate FITC- and PE-conjugated monoclonal antibodies to CD45, CD14, CD4, CD8, CD3, CD19, CD45RA, TCR-αβ or TCR-γδ. The cells were washed and resuspended in 0.5 ml of PBS, 0.1% sodium azide and subjected to FMF analysis. A total of 10,000 cells were counted and the frequency and the mean density of each cell surface marker were determined using appropriate software (FACScan; Becton Dickinson).

Cytokine mRNA Detection by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Assay

PBMCs were plated in 24-well plates at a concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 2% ABS and 2% TCH, 4 n*M* *L*-glutamine, 25 m*M* HEPES (pH 7.2), 5×10^{-5} *M* 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin

and 0.25 mg/ml amphotericin B in the presence of irradiated PBMCs plus T9 antigen (4 μg/ml) or concanavalin-A (10 μg/ml, Con A; Sigma). Thirty milliliters of heparinized blood was centrifuged at 2,500 rpm for 10 min. After removing the plasma, an equal volume of red blood cells (RBC) lysis buffer (NH_4Cl 7.47 g/l, Tris 2.05 g/l, pH 7.2) was added. The samples were mixed for 10 min, and then stood for 5 more min. After centrifugation, the supernatant was discarded and equal volume of RBC lysis buffer was added. This procedure was repeated 3 times. Finally, about 1.5 ml of WBC concentrate was yielded. The samples were aliquoted and stored at −70°C before use. RNA was extracted by acid-guanidinium thiocyanate method [8]. The RNA pellet was washed once in 75% alcohol, then the RNA pellet was dried in a vacuum for 5–15 min. The RNA pellet was washed once in DEPC-treated water. RNA in DEPC-treated water was mixed with oligo-dT primer, dNTPs and reverse transcriptase for synthesis of single-stranded cDNA. An aliquot (3 μl) of the RT reaction was adjusted to contain 1 μl of primers, 2 μl of each 10 m*M* deoxynucleotides (dATP, dCTP, dGTP and dTTP) (Pharmacia, Inc., Piscataway, N.J., USA), 5 μl of 10 × PCR buffer (100 m*M* Tris-HCl pH 8.3, 500 m*M* KCl, 25 m*M* $MgCl_2$, 0.01% gelatin (w/v) and 0.2 unit of Taq DNA polymerase (Bethesda Research Laboratory, Gaithersburg, Md., USA). The PCR procedures included: (1) heat to 94°C and maintain at 94°C for 45 s to denature; (2) cool to 60°C and maintain at 60°C for 45 s to anneal DNA with primers; (3) heat to 72°C and maintain at 72°C for 2 min to extend DNA chain. Nucleotide sequences of 5′ and 3′ primers used for the PCR amplification were as follows:

IL-2
5′ primer, 5′-CATTGCACTAAGTCTTGCACTTGTCA-3′,
3′ primer, 5′-CGTTGATATTGCTGATTAAGTCCCTG-3′;

IL-3
5′ primer, 5′-ATGAGCCGCCTGCCCGTCCTG-3′,
3′ primer, 5′-GCGAGGCTCAAAGTCGTCTGTTG-3′;

IL-4
5′ primer, 5′-ATGGGTCTCACCTCCCAACTGCT-3′,
3′ primer, 5′-CGAACACTTTGAATATTTCTCTCTCAT-3′;

IL-10
5′ primer, 5′-AAGCTGAGAACCAAGACCCAGACATCAAGGCG-3′,
3′ primer, 5′-AGCTATCCCAGAGCCCCAGATCCGATTTTGG-3′;

IFN-γ
5′ primer, 5′-GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC-3′,
3′ primer, 5′-CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG-3′.

In vitro Augmentation of the Production of Anti-T9 Antibodies

To further separate autologous B cells, PBMCs (1 × 10^7 cells) of the patient (WMS) were resuspended in 5 ml RPMI 1640 medium (Biochrom KG, Berlin, Germany) with 10% FCS (Hyclone Lab., Utah, USA). One hundred milliliters of washed Dynabeads (anti-CD2, M-450) (Dynal Inc., Oslo, Norway) was added into a 15-ml tube and mixed well with PBMCs. The cells were incubated at 2–4°C for 30 min with gentle tilt rotation. Five more milliliters of cold 0.1% BSA/PBS was added immediately before the test tube was put in a magnetic particle concentrator (MPC; M-6; Dynal Inc.). The tube was kept in the MPC for 2 min and the remaining cells were pipetted into another flask, and cultured overnight in 5% CO_2 incubator at 37°C. Phenotypic analysis of these purified cells demonstrated that the percentage of T cells was less than 5% after treatment. The T-depleted cells with the concentration of 2.5 × 10^5 cells/well were cultured with 1 × 10^5 cells/well of the T-cell clones. To determine T9 or core-specific antibody in the supernatants, the microtiter plates with 0.5 μg T9 or core antigen in 100 ml carbonate coating buffer per well were incubated at 4°C overnight. The microtiter plates were washed twice with 1 × PBS containing 0.05%. Tween 20. Then we blocked the plates with blocking solution (1% BSA-0.05% Tween 20 in 1 × PBS) and washed 3 times after the reaction. We added a 100-μl sample and washed 6 times after the reaction, and then added 100 ml anti-mouse IgG antibody and washed 6 times after the reaction. The three above mentioned reactions were all performed at 37°C for 45 min and room temperature for 15 min. Finally, 100 μl of ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) substrate solution was set at room temperature in about 30 min and the reaction stopped with 100 ml of 5% SDS. We read the plates with a microplate reader. The results of T9 or core-specific antibody were presented as ELISA units (EU/ml) compared to human control serum.

Antigen-Specific MHC-Restricted Proliferative Response

Heparinized peripheral blood was collected from the patient and mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia). EBV transformation of B cells was performed by adding 1 ml of B95-8 cell culture supernatant diluted in 4 ml of RPMI 1640 supplemented with 20% FCS and 2 μg/ml cyclosporin A (Novartis/Basel, Switzerland) with the cell concentration of 4 × 10^6 cells/ml. The cells were then incubated and the growth condition followed up. After establishment, EBV-LCLs were frozen and stored in a liquid nitrogen tank. HLA types of these EBV-LCLs were determined using Terasaki Oriental HLA typing kits (One Lambda Inc., Canoga Park, Calif., USA).

Results

Dose-Dependent Response of T Cells to T9 Antigen

Two weeks after coculture with T9 antigen plus IL-2, PBMCs from the patient were stimulated with irradiated PBMCs plus different concentrations of T9 antigen. The results of T-cell proliferative responses to different doses of T9 antigen are depicted in figure 1 and the optimal concentration of maximal proliferative response to T cells after T9 antigen stimulation was between 4 and 8 μg/ml. The proliferative response declined after increasing T9 antigen to 16 μg/ml.

Establishment of T9-Specific T-Cell Clones

Totally, six T9-specific T-cell clones were derived from our patient and all have been maintained in our laboratory for more than 1 year. To test antigen specificity of our cloned T-cell lines, T cells were incubated with irradiated APCs with or without T9 antigens. Our data showed these T-cell clones specifically responded to T9 antigen, but not to the core protein of HCV or to the tetanus toxoid antigen (fig. 2). All these clones were stained positively with CD4, but negatively with CD45RA, therefore suggesting that they were of memory type T-helper cells. In addition, all these T-cell clones expressed αβ TCR on their surface.

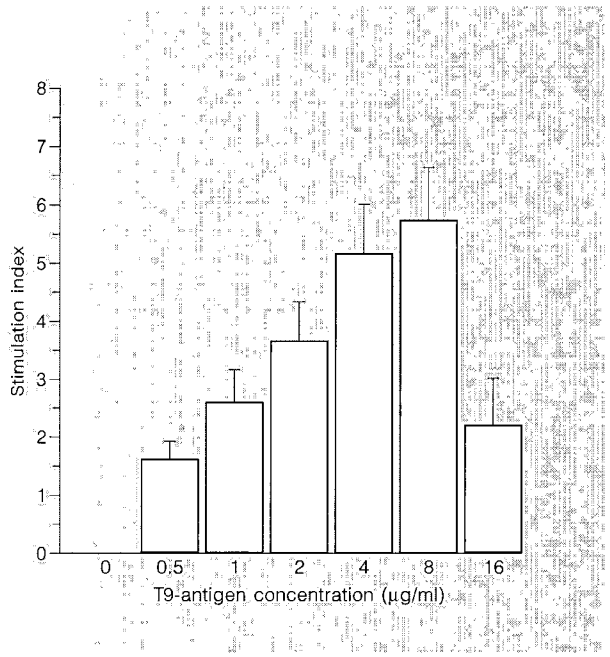


Fig. 1. Titration curve of T9 antigen dose of proliferative responses of T cells after one cycle of antigen stimulation. A maximal proliferative response was reached with the antigen concentration of 4 or 8 μg/ml. The cpm value of T cells without antigen was 524 ± 35 in this assay.

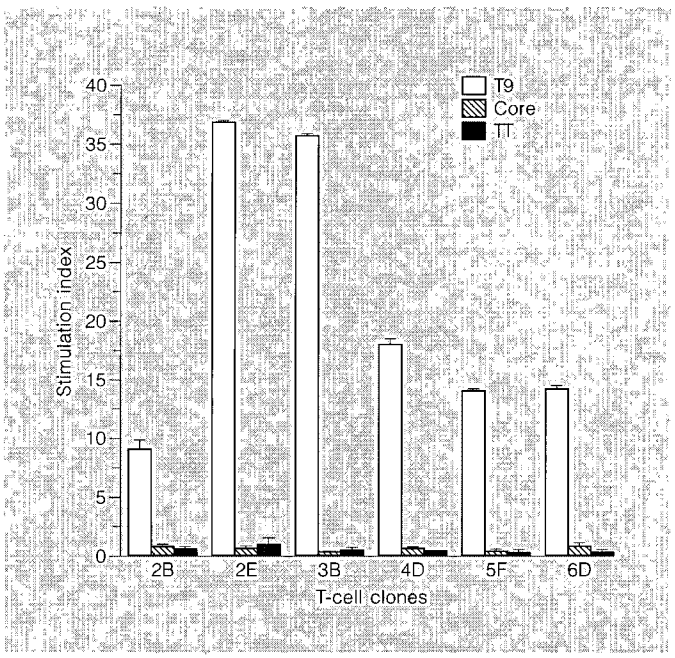


Fig. 2. Proliferative responses of cloned T-cell lines to hepatitis C T9, core and tetanus toxoid (TT) antigens. The T9-specific T-cell clones responded to T9 antigen only, not to the other two antigens. The background values of T cells without antigen stimulation were between 550 and 1,246 cpm in this assay.

Cytokine Production Pattern of T9-Specific T-Cell Clones

Cytokine mRNA expression was determined for the six T-cell clones. Data suggested 5 out of our 6 clones produced IL-2 and γ-IFN, but not IL-4 or IL-5 cytokines (table 1). However, one particular T-cell clone (4D) expressed IL-2, γ-IFN and IL-4 mRNA after stimulation. The data suggested these T9-specific T-cell clones are TH_1 or TH_0 in nature.

Anti-T9 Antibody Augmentation Ability of T-Cell Clones

After 5 days' culture, the supernatant was collected and assayed for antibody production. Our data showed these T-cell clones helped autologous B cells (WMS) in producing anti-T9 antibody in vitro, but not anti-C190 antibody (fig. 3). These results suggested all these T-cell clones had antigen-specific proliferative response and specific antibody augmentation ability.

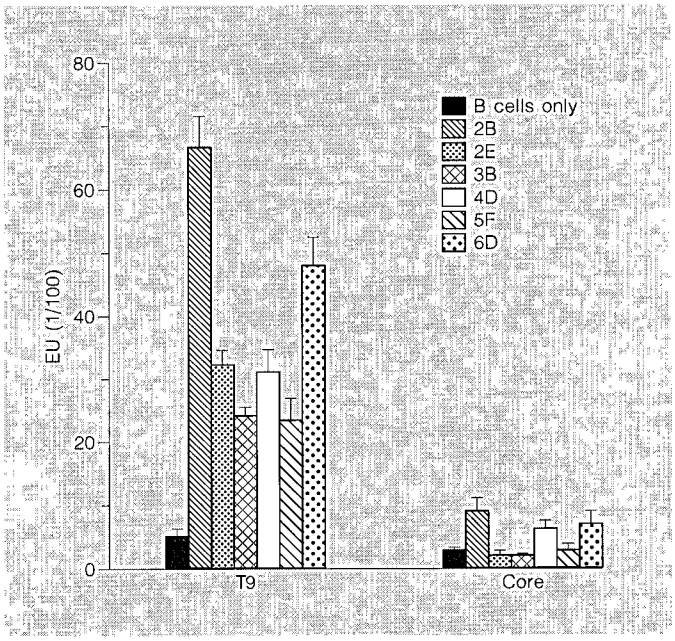
MHC Restriction of T-Cell Proliferative Responses

To further investigate the MHC-restricted pattern of all these T-cell lines, we have developed about 25 EBV-LCLs from different patients and all these EBV-LCLs were HLA-typed. Three other EBV-LCLs of partially matched HLA type (HSY, TDS, CCT) were used for MHC-restricted proliferative study and the results are shown in table 2. The data suggested that T9 antigen-spe-

Table 1. Patterns of cytokine mRNA expression of the cloned T-cell lines by RT-PCR

	Cytokines					
	2B	2E	3B	4D	5F	6D
IFN-γ	+++[a]	+	+	++	+	+
IL-2	++	+	–	++	++	++
IL-3	++	++	++	+++	+++	++
IL-4	–	–	–	+	–	–
IL-10	–	–	–	–	–	–

[a] The density of cytokine mRNA was compared to that of β-actin and the ratio of cytokine to β-actin was calculated: +++, strong: the ratio was >0.5; ++, moderate: the ratio was between 0.25 and 0.5; +, weak: the ratio was <0.25; –, negative.



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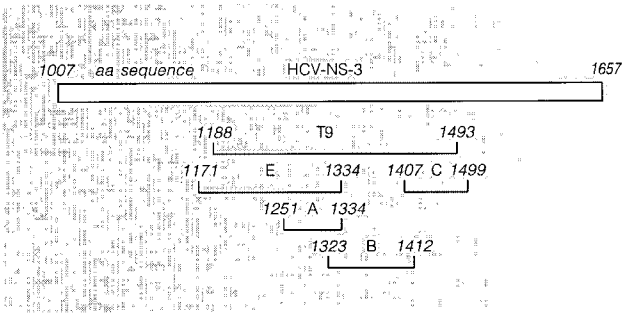

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Fig. 3. In vitro antibody production by autologous B cells incubated with six T9 antigen-specific T-cell clones. After 5 days' culture, B cells produced antibody against T9 antigen but not against the core antigen.

Fig. 4. Location of total 5 recombinant antigens in HCV-NS-3 region. Amino acid sequences are italicized.

Table 2. EBV-transformed lymphoblastoid cell lines used for MHC-restricted proliferative assay

Patients	DR	DQ	Stimulation index					
			2B	2E	3B	4D	5F	6D
WMS	DR17,DR52	DQ2,DQ6	8.9[a]	36.8	35.6	22.8	14.1	5.7
HSY	DR16,DR53	DQ6,DQ8	1.4	1.3	0.9	0.8	0.5	0.5
TDS	DR9,DR53	DQ1,DQ2	18.3	7.5	38.7	19.1	4.2	4.8
CCT	DR11,DR52	DQ4,DQ7	1.1	1.6	0.8	0.5	1.1	0.5

[a] The background values of T cells without antigen stimulation were between 850 and 1,560 cpm in this assay.

cific proliferative responses are most likely restricted to HLA DQ2 antigens.

Purification of Recombinant Proteins and Proliferative Responses of T-Cell Clones to These Proteins

Four proteins, E (aa 1175–1334), A (aa 1251–1334), B (aa 1323–1412) and C (aa 1407–1499), encoded by NS-3-region internal clones were expressed and tested for antigen-proliferative response (fig. 4). Except clone 4D, all the cell clones responded to proteins T9 and C (fig. 5). Since the clone 4D responded to T9, E and A, but not to proteins B and C, it most likely recognized antigenic determinants located inside protein A. In addition, clone 4D was the only T cell bearing a TH_0 pattern.

Discussion

HCV has been shown to be responsible for most of the parenterally transmitted non-A, non-B hepatitis viral infections after identification of viral genome [1, 6, 18]. One important yet puzzling problem is the very high likelihood of chronicity after acute infection, and a substantial proportion of the chronic infection will develop cirrhosis and hepatoma. The virus differs from the other hepatitis viruses in the very low virus load in the host, the level is so low that it could only be detected by the sensitive PCR assay [19, 21]. In addition, hypermutation has been found to occur frequently in the E2 region of HCV genome, possibly resulting in the lack of effective neutralizing antibody after infection [22]. Several mechanisms such as

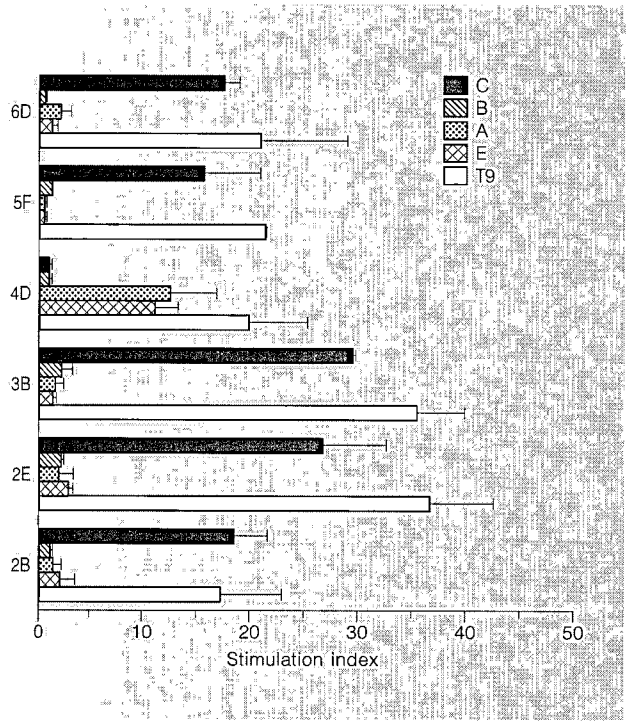

Fig. 5. Proliferative responses of cloned T-cell lines to recombinant proteins E, A, B and C. All cell clones responded to protein T9 and C except clone 4D which responded to T9, E and A. The findings suggested clone 4D most likely recognized antigenic determinant located inside protein A. The background values of T cells without antigen stimulation were between 720 and 2,020 cpm in this assay.

scape mutation of CTL epitopes, lack of effective neutralizing antibody and dysregulation of T-helper cells have been suggested to play important roles in the chronicity of HCV infection [12, 28].

Studies on hepatitis B virus (HBV) suggested that mutations within the CTL epitopes may be exploited by HBV to escape the protective immune responses which are critical for viral clearance [5]. Several studies concerning cytotoxic T-cell responses isolated from patients infected with HCV have been reported and epitopes recognized by these T cells are well documented [15, 20, 26]. However, there is no convincing evidence supporting the notion of escape mutation of the CTL epitope during the disease progression of HCV infection. In the meantime, HCV-specific T-helper cells were inadequately addressed before [13, 25, 29]. NS-3 protein with serine protease activity has been found to be required in the processing of HCV polyprotein [2, 27]. It is well known that NS-3 protein is one of the major B-cell antigens during HCV infection [17]. The data we presented here clearly indicated at least two different T-cell antigenic determinants within the NS-3 region. Our previous findings on B-cell epitopes in this region demonstrated multiple antigenic determinants recognized by the B cells [14].

It has been suggested that cytokines such as IL-12 or IFN-α/β secreted by macrophages and fibroblasts are important for antiviral immune responses during the early stage of viral infection [4]. These cytokines are essential for the development of natural killer cells, TH_1 and cytotoxic T cells which are important in viral clearance. The defect of T-helper cell responses may contribute to the absence of effective neutralizing antibody and cytotoxic T-cell response. Dysregulation of cytokines has been suggested to be critical in certain infections such as HIV and leishmaniasis [9, 10, 24]. Persistence of HCV infection has been suggested to be correlated to T-helper cell imbalance between TH_1 and TH_2, however, no definite evidence has been documented. All of the T-helper cell clones isolated in our study secreted IFN-γ and only one of them also produced IL-4. Another study by us has demonstrated that PBMCs in about 44% of HCV patients produced a significant amount of IL-4 and IL-5 after stimulation with T9 or A protein (data not shown). It has been suggested that antigen-specific TH_1 cells tend to proliferate better than TH_0 or TH_2 cells, which may account for the discrepancy between cytokine pattern of T-cell clones and PBMCs [23].

We believe further elucidation of T-helper cell response in HCV infection will provide deeper insights on understanding the mechanism of chronicity in HCV infection. Furthermore, this information might help in designing immunotherapy for HCV infection in the future. It will also be interesting to see whether or not the effect of interferon-α in the treatment of hepatitis C is related to its influence on TH_1 development.

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