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Hepatology Research 27 (2003) 18–22
Hepatology Research
www.elsevier.com/locate/ihepcom

Detection, using a novel method, of a high prevalence of cryoglobulinemia in persistent hepatitis C virus infection

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Received 18 June 2002; received in revised form 19 March 2003; accepted 26 March 2003

Abstract

To elucidate precisely the prevalence and significance of cryoglobulinemia in hepatitis C, we examined the prevalence of serum cryoglobulin (CG) among 232 consecutive hepatitis C virus carriers (23 asymptomatic carriers, 164 with chronic hepatitis, 45 with cirrhosis), 30 consecutive hepatitis B virus carriers and 100 age- and sex-matched healthy volunteers. We used a gel-diffusion procedure that detects CG with greater sensitivity and specificity than the conventional precipitation method. Among the 232 patients, 166 were tested for the presence or absence of CG by the precipitation method also, which showed 60 (36.1%) patients to be positive for CG. On the other hand, 164 of the 232 patients (70.7%) were positive for CG using the diffusion method. 5 (16.7%) of the 30 HBV carriers and 2 (2%) of the healthy volunteers also were positive for CG using the gel-diffusion procedure. CG was detected more frequently among the patients with chronic hepatitis or cirrhosis than the asymptomatic carriers. In spite of the high prevalence of CG positivity, only one patient had symptoms related to cryoglobulinemia. Positivity for CG was not related to viral serogroup, viral load or the presence of antinuclear antibody, but it was related closely to CH50: 58 of 63 (92.1%) patients with lower levels of CH50 were positive for serum CG. In conclusion, cryoglobulinemia is a very common feature of chronic hepatitis C.

Keywords: Cryoglobulinemia; Hepatitis C; Gel-diffusion procedure; Precipitation method

1. Introduction

Cryoglobulinemia frequently complicates HCV infection. Although the mechanisms which generate cryoglobulin (CG) in HCV infection remain unclear, monoclonal or polyclonal activation of B cells by viral proteins has been proposed to be an important step [1]. It also has been proved that monoclonal B cell activation, expressed as a monoclonal rheumatoid factor (RF), is driven by one common immunological mechanism among those who share a particular cross-reacting idiotype [2]. In contrast, the mechanisms of polyclonal B cell activation remain obscure.

The prevalence of cryoglobulinemia in chronic hepatitis C differs widely among various reports [3–12], and this may be attributed to the varying sensitivity of standard methods. At present, CG is detected by cold precipitation of serum samples that are collected at 37 °C. The samples are stored at 4 °C for about a week to detect cryoprecipitation. Although this method is simple, it requires significant time and a small amount of CG may be overlooked. The lack of a standard method may obscure the significance of CG in chronic hepatitis C.

We have established a gel-diffusion procedure that detects CG more sensitively and precisely than the

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1386-6346/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1386-6346(03)00189-X

conventional precipitation method [13]. Using this method, we investigated the prevalence and significance of CG among patients with chronic hepatitis C and found that positivity for CG among HCV carriers is very high.

2. Materials and methods

2.1. Patients

We analyzed prospectively 232 consecutive patients with chronic liver disease type C who visited our hospital from December 1998 to March 1999. Diagnosis of a chronic HCV infection was made by continuous serum HCV-RNA for more than 6 months. The patients included 23 asymptomatic carriers (AsC), 164 with chronic hepatitis (CH) and 45 with cirrhosis (LC). Diagnosis of an asymptomatic carrier was made by continuous normal ALT levels for more than 1 year in serum samples tested every 3–4 months. Chronic hepatitis was diagnosed by monthly blood examinations as continuous elevation of ALT for more than 6 months. Chronic hepatitis and cirrhosis were differentiated using the calculation formula of Ikeda et al. [14]. 30 consecutive patients with chronic hepatitis B virus infection and 100 healthy volunteers, whose age and sex were matched with the patients, were enrolled in the study as controls. Informed consent was taken from each patient and volunteer, based on the rules of the ethical committees in our hospitals.

2.2. Viral and serological markers

Levels of HCV-RNA were determined using an Amplicor-M kit (Roche Diagnostics, Tokyo, Japan). The serogroup of HCV was determined using a commercial ELISA kit (Tonen Chemistry, Tokyo, Japan).

To elucidate further the significance of cryoglobulinemia, levels of IgG, IgM, IgA and rheumatoid factor were determined. Because cold activation of serum complement also is found frequently in HCV infection [15,16], CH50 also was measured in each patient.

2.3. Detection of cryoglobulin

Cryoglobulin was detected using the precipitation and gel-diffusion methods. Serum samples were collected and separated at 37 °C. Samples were kept at 4 °C for 1 week for the detection of CG using the conventional cryoprecipitation method. Any cryoprecipitate detected was confirmed as CG if the precipitate dissolved after warming to 37 °C.

The procedure for the gel-diffusion method has been described previously [13]. In short, 10 μl of the serum sample collected at the time of conventional analysis was applied to a glycin-containing gel and left at 4 °C for 48 h. Visualization of a ring around the well indicated CG positivity.

2.4. Statistical analysis

The data were analyzed using the Chi-square test, Mann–Whitney *U*-test or Fisher's exact test where appropriate. *P*-values less than 0.05 were regarded as significant.

3. Results

3.1. Positivity of cryoglobulin (Table 1)

The conventional precipitation method showed that 63 of the 166 HCV carriers (36.1%) were positive for CG. On the other hand, 164 of 232 patients (70.7%) were positive for cryoglobulin using the diffusion method. All but one patient who tested positive for cryoglobulin using the precipitation method also was positive by the diffusion method. 5 of the 30 patients (16.7%) with HBV infection and 2% (2/100) of the healthy volunteers were positive for CG by the diffusion method.

Table 1 compares the positivity for CG as detected by the two procedures. We examined 166 samples in total. 59 of 60 samples (98.3%) that were positive for CG by the precipitation method also were positive by the gel-diffusion method. 22 of 23 samples (95.7%) that were negative for CG by the gel-diffusion method also were negative by the precipitation method. These data show that the gel-diffusion method can detect CG with greater sensitivity and specificity than the precipitation method ($P < 0.0001$). Therefore, we carried out the following CG analysis using data from the gel-diffusion method.

3.2. Relationship between progression of disease and CG positivity (Table 2)

Table 2 shows the positivity for CG (using the gel-diffusion method) among each subset of chronic hepatitis C. Serum CG was detected in 30.4% of AsC, 81.9% of CH and 97.8% of LC patients. Positivity for CG increased with the progression of disease ($P < 0.0001$).

3.3. Relationship between biochemical, serological and viral markers and CG positivity (Table 3)

The correlation between positivity or negativity for CG and biochemical, serological and viral markers among patients with chronic hepatitis C is shown in Table 3. As shown in Table 3, levels of γ-GTP and hyalulonic acid were higher in CG-positive patients than CG-negative patients (γ-GTP: 51.7±45.5 IU/l vs. 37.3±

Table 1
Positivity of cryoglobulin by the precipitation method and the gel-diffusion method

	Positive by gel-diffusion method	Negative by gel-diffusion method	Total
Positive by gel-diffusion method	59 (98.3%)	1 (1.7%)	60 (36.1%)
Negative by gel-diffusion method	84 (79.2%)	22 (20.8%)	106 (63.9%)
Total	143 (86.1%)	23 (13.9%)	166

$P < 0.001$ by Chi-square test.

Table 2
Relation between progression of disease and CG positivity

Disease subset	Positive by gel-diffusion method	Negative by gel-diffusion method	Total
Asymptomatic carrier	7 (30.4%)	16 (69.6%)	23
Chronic hepatitis	133 (81.1%)	31 (18.9%)	164
Liver cirrhosis	44 (97.8%)	1 (2.2%)	45
Total	184 (70.7%)	48 (29.3%)	232

31.5 IU/l, $P = 0.04$; hyalulonic acid: 148.4±379.0 g/l vs. 51.5±60.8 g/l, $P = 0.004$), although levels of ALT did not differ (54.0±40.3 IU/l vs. 49.5±41.7 IU/l, $P = 0.69$). As shown in Table 3, levels of HCV-RNA did not differ between the CG-positive patients and CG-negative patients ($10^{5.48}$ copies/ml vs. $10^{5.43}$ copies/ml, $P = 0.47$). Positivity for CG also did not differ between patients with serogroup 1 and those with serogroup 2 HCV (86.9% vs. 91.9%, $P = 0.41$; Table 3).

Table 3
Relation between viral markers and CG positivity

	Cryoglobulin-Positive	Cryoglobulin-Negative	P
	($n = 143$)	($n = 23$)	
Biochemical and serological tests			
ALT (IU/l)	54.0±40.3	49.5±41.7	0.69
γ-GTP (IU/l)	51.7±45.5	37.3±31.5	0.04
Hyalulonic acid (g/l)	148.4±379.0	51.5±60.8	0.004
	($n = 143$)	($n = 17$)	
HCV-RNA			
HCV-RNA (log-KIU/ml)	2.48±0.75	2.43±0.91	0.47
	($n = 140$)	($n = 19$)	Total
HCV serogroup			
Serogroup 1	106 (86.9%)	16 (13.1%)	122
Serogroup 2	34 (91.9%)	3 (8.1%)	37
Total	140	19	159

3.4. Relationship between cryoglobulinemia-related signs and CG positivity (Table 4)

The correlation between signs related to essential cryoglobulinemia (purpura, proteinuria, hematuria) and positivity or negativity for cryoglobulin is shown in Table 4. The signs could be checked in 134 CG-positive and 17 CG-negative patients. All the patients who showed such signs were positive for cryoglobulin. Hematuria was found in 11 (7.3%), and proteinuria in 6 (4.0%), of 151 patients. Skin manifestation was found only in one patient (0.7%). Positivity for the three signs did not differ between CG-positive and CG-negative patients (purpura, proteinuria and hematuria: $P = 0.99$, 0.99 and 0.61, respectively).

3.5. Relationship between immunological markers and CG positivity (Table 5)

The correlation between serum immunological markers and positivity or negativity for cryoglobulin is shown in Table 5. All patients whose sera were reactive for rheumatoid factor were positive for cryoglobulin, although the positivity for rheumatoid factor did not differ between the CG-positive and CG-negative groups

Table 4
Relation between cryoglobulinemia-related signs and CG positivity

CG-related sign	Cryoglobulin-negative	Total	P
Purpura	0/17	1/151 (0.7%)	0.99*
Proteinuria	0/17	6/151 (4.0%)	0.99*
Hematuria	0/17	11/151 (7.3%)	0.61*

* Fisher's exact test.

Table 5
Relation between immunological markers and CG positivity

Immunological marker	Cryoglobulin-positive	Cryoglobulin-negative	Total	P
Antinuclear antibody	22/177 (12.5%)	2/32 (6.3%)	24/209 (11.5%)	0.31*
CH50	58/126 (46.0%)	5/23 (21.7%)	63/149 (42.3%)	0.03*
Rheumatoid factor	16/142 (11.3%)	0/26	16/168 (9.5%)	0.13*

* Chi-square test.

($P = 0.13$). Moreover, 58 of 126 (46.0%) CG-positive patients had decreased complement activity, a higher prevalence than the CG-negative patients (5 out of 23, 21.7%) ($P = 0.03$). 58 of 63 (92.1%) patients with lower levels of CH50 were positive for serum CG. Anti-nuclear antibody positivity did not differ between the CG-positive and CG-negative groups (12.5% vs. 6.3%, $P = 0.31$).

4. Discussion

There have been several reports examining the extra-hepatic manifestations that may accompany HCV infection [4,5,9–11]. Cryoglobulinemia was the most frequent clinical manifestation in each study. The prevalence of cryoglobulinemia among HCV carriers is reported to be between 21 and 59%. This high prevalence, irrespective of ethnic origin, suggests that cryoglobulinemia is a common phenomenon in individuals with HCV infection. Our study shows that CG may be found in a very high proportion of HCV carriers using a gel-diffusion method.

As shown in Table 1, the gel-diffusion method is more sensitive than the conventional precipitation method for the detection of CG. Although the gel-diffusion method missed one serum sample that was found to be CG-positive using the precipitation method, it may be said that the gel-diffusion method is superior to the precipitation method.

Table 2 shows that CG positivity increases with the progression of disease, which is compatible with previous reports [7]. Furthermore, CG is detected even during the asymptomatic stage in about one-third of patients. Recent studies have shown that the number of CD5+ B-cells and the expression of CD81 on B-cells are increased in chronic HCV carriers compared with healthy controls. Furthermore, they are closely associated with the production of rheumatoid factor and mixed cryoglobulins [17,18]. Because HCV can infect peripheral blood mononuclear cells [19–26] and CD81 is a putative co-receptor of HCV [27,28], HCV-associated CG production may start even at an early stage of chronic infection. A longer duration of infection may lead to a higher probability of CG, as observed in our patients with cirrhosis.

As shown in Table 3, levels of HCV-RNA and viral serogroup did not affect CG positivity. The major HCV serogroups found in Japan are serogroups 1 and 2. Furthermore, the number of studied patients is not sufficiently large. Although our data show that CG is common irrespective of viral type and amount, further studies are necessary to examine our hypothesis.

As shown in Table 4, skin manifestations, proteinuria and hematuria were not observed more frequently among the CG-positive groups than the CG-negative groups. The lack of a difference may be attributable to the low frequency of the three symptoms. Most of chronic hepatitis C patients with cryoglobulinemia are asymptomatic, as shown in previous reports [7–9].

Table 5 shows that CG positivity is related closely to low complement activity. Although not statistically different, rheumatoid factor was detected exclusively in CG-positive patients. Because CG consists of immunoglobulin that has rheumatoid factor activity, it is logical that CG and RF are correlated strongly. The low frequency of positivity for the two markers probably is attributable to the sensitivities of the two tests.

It was reported that cold activation of complement was observed frequently among HCV carriers [15,16]. It has been proposed that IgG–IgM rheumatoid factor (RF) complex induced by HCV may be the main causal factor for cold-dependent activation of complement in patients with rheumatic disease [29], which suggests that cold activation is associated closely with cryoglobulinemia. Our study shows that positivity for CG using the gel-diffusion method is higher than that of hypocomplementemia. One study of blood donors has shown that cold activation of complement is a phenomenon observed frequently among HCV carriers.

We have shown already that the gel-diffusion method may be used for analysis of CG. Melting the gel cut from the area with the CG ring may lead to a simple method for calculating the amount of CG. Diffusion with anti-immunoglobulin antibodies enables us to determine the components of CG. The gel-diffusion method is a useful tool for large-scale study.

In conclusion, cryoglobulinemia is a common extra-hepatic manifestation of HCV infection, especially in patients with liver injury. The gel-diffusion method is useful to study cryoglobulinemia and may substitute for the precipitation method.

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Original Paper

Intervirology

Intervirology 2003;46:56–65
DOI: 10.1159/000068126

Received: October 6, 2002
Accepted after revision: October 12, 2002

Autoantibodies to CD69 in Patients with Chronic Hepatitis Type C: A Candidate Marker for Predicting the Response to Interferon Therapy

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Key Words
Chronic hepatitis C · Autoimmunity · Autoantibody · CD69 · Hepatitis C virus · Interferon

Abstract
Objective: To understand the autoimmunity associated with chronic hepatitis C (CHC), we investigated autoantibodies (autoAbs) to CD69. ***Methods:*** With this aim, we tested the reactivity of serum samples from patients with CHC and asymptomatic carriers of hepatitis C virus (HCV), as well as from patients with chronic hepatitis B (CHB) and autoimmune hepatitis (AIH), to recombinant CD69 molecules. ***Results:*** Frequencies of anti-CD69 autoAbs were 38.7% in CHC, 15.8% in AIH and 12.3% in CHB. None of the tested asymptomatic HCV carriers had autoAbs to CD69. It is important clinically that the presence of anti-CD69 autoAbs was found to be associated with a poor response to interferon-α (IFN-α) therapy. In the epitope analysis, multiple epitopes were mapped on CD69, indicating antigen-driven production of the autoAbs. ***Conclusion:*** We evidenced existence of anti-CD69 autoAbs in patients with CHC, and found that the anti-CD69 autoAb may have potential for predicting responses to IFN-α therapy.

Introduction

Infection with hepatitis C virus (HCV) gives rise to serious liver disorders. Specifically, about 80% of patients with acute hepatitis due to HCV become chronic HCV carriers [1]. A substantial portion of chronic HCV carriers develop chronic hepatitis which progresses steadily and finally leads to liver cirrhosis and hepatocellular carcinoma [2–11]. An inadequate and/or insufficient immune response to HCV allows persistence of the virus and thus also of chronic liver injury, although the precise mechanisms remain unclear [12–17]. In this regard, autoimmune phenomena, like autoantibodies (autoAbs) to nuclear proteins, smooth muscle and thyroid, are often observed in patients with chronic hepatitis C (CHC) [18–21]. Such autoimmune phenomena may reflect inade-

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quate immune responses to HCV and thus could be a clue to understanding immune responses to HCV. Further, the autoimmunity found in HCV infection may play a role in chronic inflammation of the liver. Therefore, the autoimmunity in HCV infection should be investigated in more detail. In this context, the anti-lymphocyte antibody (ALA) is one of the autoAbs which should be investigated in HCV infection, since ALA is detected frequently in systemic inflammatory diseases [22, 23]. However, to our knowledge, nor report is currently available that describes ALA in patients with CHC or HCV infection. Therefore, in the present study, we investigated the existence of ALA, in particular autoAbs to CD69, one of the activation surface marker molecules of T cells. We chose this approach because the existence of autoAbs to CD69, detected in 38% of patients with rheumatoid arthritis (RA) in our previous study [24], was related with the severity of RA, and profiles of the autoimmunity in CHC and RA have similar aspects, such as frequent detection of rheumatoid factors. Further, in both conditions, the inflamed tissue, liver in CHC and synovium in RA, is infiltrated with CD69-positive T cells [25–28].

Specifically, we prepared recombinant CD69 molecules and tested their reactivity to serum samples from patients with CHC as well as from asymptomatic carriers, patients with chronic hepatitis B (CHB) and autoimmune hepatitis (AIH) and healthy donors. We found that 38.7% of the tested CHC patients possessed anti-CD69 autoAbs, whereas only 12.3, 15.8 and 0% of the CHB patients, AIH patients and asymptomatic HCV carriers, respectively, possessed anti-CD69 autoAbs. Further, the CHC patients with anti-CD69 autoAbs were found to be resistant to interferon-α (IFN-α) therapy. Our data suggest that mechanisms for the production of anti-CD69 autoAbs may be involved in the pathological process of CHC and that anti-CD69 autoAbs would be useful for predicting the effectiveness of IFN-α therapy.

Patients and Methods

Patients, Liver Specimens and Serum Samples

Serum samples were obtained at the time of liver biopsy with informed consent from 127 patients who were admitted to the St. Marianna University hospital during the period from August 1994 to December 1999. They consisted of 61 patients with CHC (43 males and 18 females; mean age 47.5 years, ranging between 23 and 77 years), 47 patients with CHB (32 males and 15 females; mean age 37.7 years, ranging between 20 and 65 years), 19 patients with AIH (7 males and 12 females; mean age 50.1 years, ranging between 26 and 78 years) and 19 asymptomatic HCV carriers as defined by normal serum aspartate or alanine aminotransferase levels on periodical blood examination (7 males and 12 females; mean age 50.1 years, ranging between 26 and 78 years). The mean HCV RNA level was 637.2 KIU/ml. All of the 61 patients with CHC underwent liver biopsy and were subsequently treated with IFN-α (504 MU in total). Histological activity of hepatitis C was evaluated using the biopsied liver samples by the scoring system reported by Knodell et al. [29] or the METAVIR algorithm [30]. Serum samples from age- and sex-matched healthy donors were used as controls for each disease category. The research was carried out in accordance with the human experimentation guidelines of our institution and the Helsinki Declaration.

Viral Markers

Levels of HCV RNA were determined by quantitative reverse transcriptase polymerase chain reaction assay (Ampricor HCV Monitor Test Kit, Roche Diagnostic Systems, Branchburg, N.J., USA). The HCV serotyping was performed using a commercial assay kit (Immuncheck F-HCV Gr, Kokusai International Co., Hyogo, Japan).

Recombinant CD69 Fusion Proteins

The plasmids for production of the CD69 fusion proteins used in this study were described in our previous study [24]. Briefly, pMAL-CD69^{full}, pMAL-CD69^{F1}, pMAL-CD69^{F2}, pMAL-CD69^{F3} and pMAL-CD69^{F3d} were used for transformation of *Escherichia coli*. The CD69 proteins produced as fusion proteins with maltose-binding protein (MBP) cover the entire CD69-full (amino acid residues 1–199), CD69-F1 (amino acid residues 1–98), CD69-F2 (amino acid residues 91–146), CD69-F3 (amino acid residues 138–199) and CD69-F3d (amino acid residues 184–199), respectively (fig. 1). Each of the fusion proteins was purified using amylose resin columns (New England Biolabs, Beverly, Mass., USA) according to the manufacturer's instructions. pMAL-C, an expression vector producing MBP alone, was also used as a control.

Enzyme-Linked Immunosorbent Assay

Ninety-six-well microtiter plates (Cook Dynatech, Alexandria, Va., USA) were coated by placing in each well 50 µl of 10 µg/ml purified CD69 fusion proteins or MBP (as a control) in a carbonate buffer (50 m*M* sodium carbonate, pH 9.6) at 4° for 8 h. After washing with phosphate-buffered saline (PBS) containing 0.1% Tween 20 5 times, the plates were incubated in 3% bovine serum albumin (BSA)-PBS-0.1% Tween 20 at 4° overnight. The plates were washed in PBS-0.1% Tween 20 10 times. To absorb the reactivity of the serum sample to bacterial proteins and MBP, each serum sample was incubated in 3% BSA-PBS-0.1% Tween 20 with 100 µg/ml bacterial lysate containing nonrecombinant pMAL products at room temperature for 2 h in advance. Fifty microliters of each serum sample was placed in each well at room temperature for 2 h. After washing 10 times with PBS-0.1% Tween 20, the plates were incubated in 4,000-fold diluted peroxidase-conjugated goat anti-human IgG antibodies at room temperature for 1 h, and then washed 10 times with PBS-0.1% Tween 20. Color development was achieved by adding 100 µl of peroxidase substrate, which consisted of 0.04% *o*-phenylene-diamine and 0.01% hydrogen peroxide in 0.1 *M* citrate-0.2 *M* N_2HPO_4, pH 5.0, to each well. After 15 min, the color reaction was stopped by adding 50 µl of 6 *N* H_2SO_4 to each well. The absorbance was measured with an enzyme-linked immunosorbent assay (ELISA) microplate photometer at 492 nm and 630 nm. Each sample was measured in triplicate.

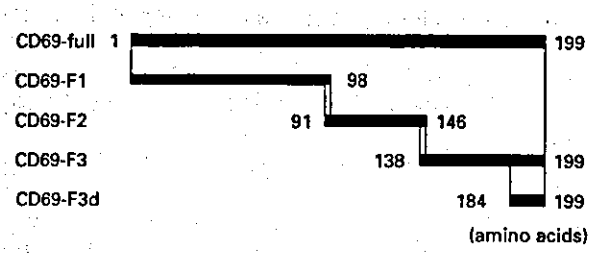

Fig. 1. Map of the recombinant CD69 proteins used in this study. Each of CD69-full, F1, F2, F3 and F3d was produced as a fusion protein with MBP using the plasmid vector pMAL.

The reactivity to the fusion proteins was expressed in units according to the following formula: binding units = [OD_{sample}/(mean $OD_{healthy}$ + 3 SD of normal sera)] × 100, where OD is the optical density. To obtain OD_{sample} and $OD_{healthy}$, the OD value of MBP was subtracted from that of the fusion protein in each assay. According to this formula, we defined the cutoff point as 100 binding units.

Western Blot Analysis

Western blotting was performed as described previously [31]. Briefly, 5 μg of each purified fusion protein or MBP (as a control) was separated by 10% SDS-PAGE and then transferred onto a nitrocellulose membrane. After blocking with PBS containing 1% BSA and 0.1% Tween 20 for 2 h and washing in PBS with 0.1% Tween 20 for 5 min 3 times, each membrane was then incubated with goat anti-human CD69 antibody (Santa Cruz Biotechnology, Santa Cruz, Cal-

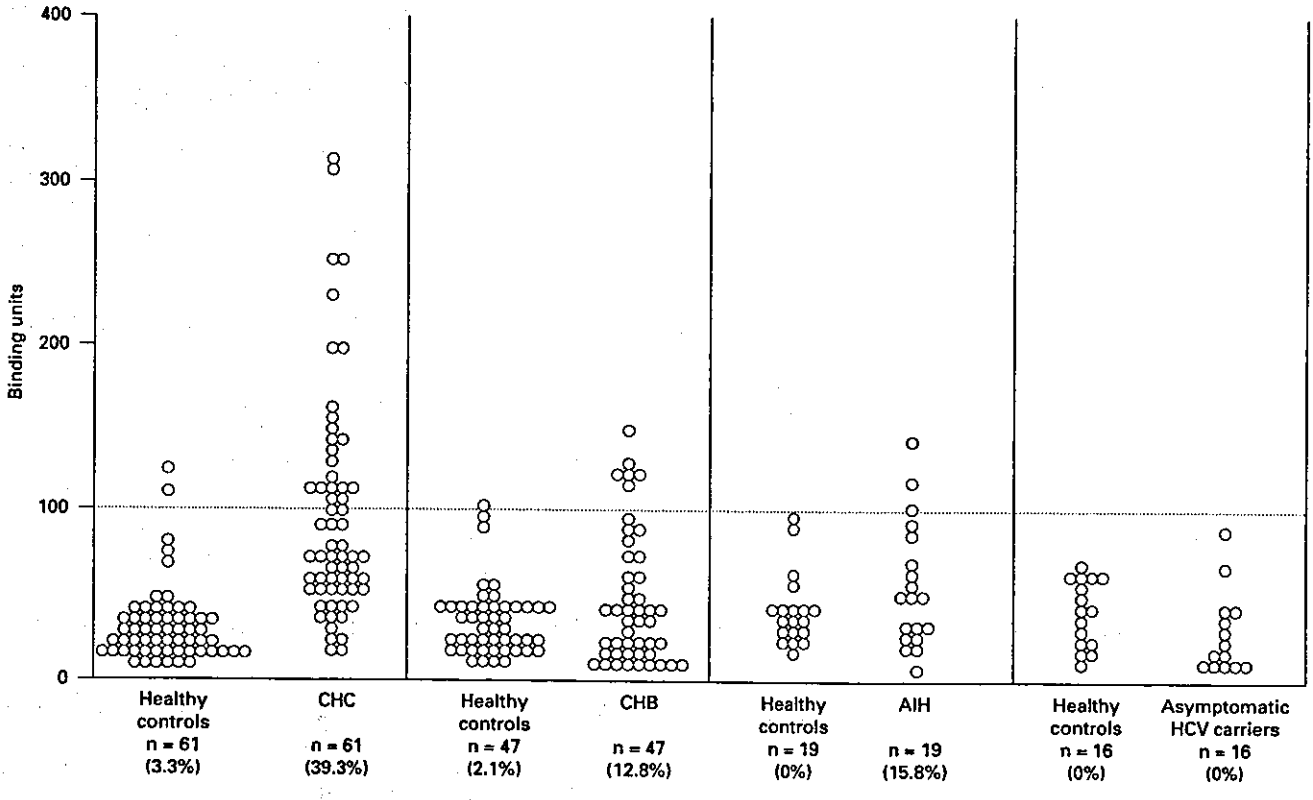

Fig. 2. Anti-CD69 autoAbs detected by ELISA. AutoAbs to the recombinant CD69-full protein (fig. 1) were titered by ELISA. Serum samples were diluted at 1:500. Identical numbers of serum samples from sex- and age-matched healthy controls were also titered for each of the disease categories. The OD value of each sample is indicated by binding units, calculated according to the formula given in Patients and Methods. The dotted line indicates the positive cutoff value of 100 binding units. The positivity of anti-CD69 autoAbs is indicated by percentages. n = Numbers of samples tested.

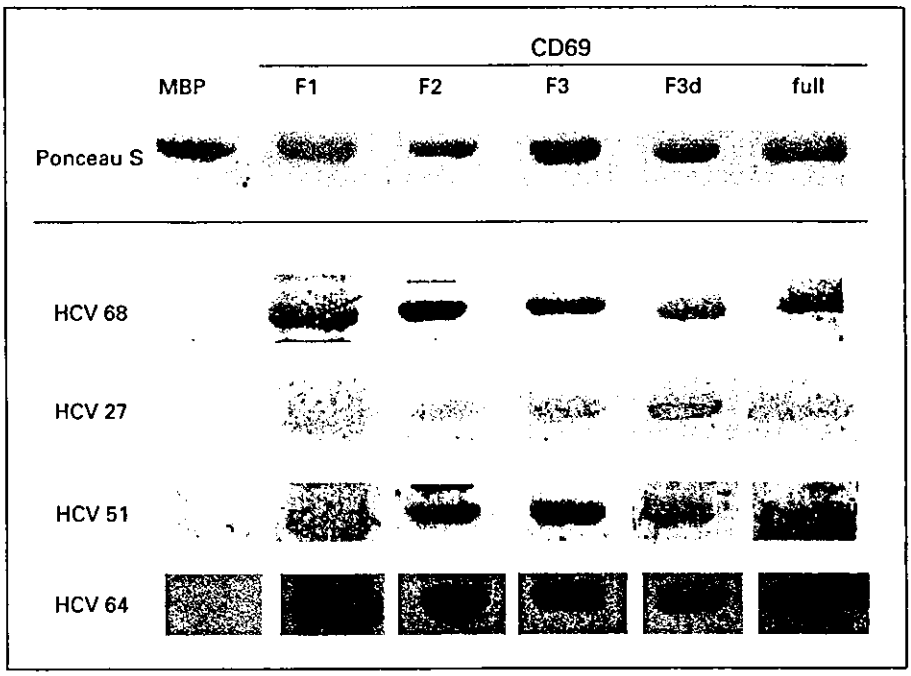

Fig. 3. Representative results of Western blotting. The recombinant CD69-full protein and MBP as a control were separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were reacted with serum samples diluted at 1:100 and then with horseradish peroxidase-labeled anti-human IgG antibodies. The bound autoAbs were visualized with diaminobenzidine.

if., USA), goat anti-MBP antibody (Santa Cruz Biotechnology) and each serum sample for 2 h. Before the membrane was incubated with the serum sample, the serum sample was diluted at 1:100 with PBS containing 1% BSA and 0.1% Tween 20 and preincubated with 2 mg/ml bacterial lysate containing nonrecombinant pMAL-c product for 2 h at room temperature. Following membrane incubation, the membrane was washed 5 times in PBS with 0.1% Tween 20, and the bound antibodies were reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (Medical & Biological Laboratories, Nagoya, Japan) or goat anti-human IgG (Zymed Laboratories, San Francisco, Calif., USA) diluted at 1:3,000–10,000 with PBS containing 1% BSA and 0.1% Tween 20 for 1 h. The bound antibodies were visualized with diaminobenzidine.

Statistical Analysis

Laboratory parameters are expressed as the mean ± SEM. The Mann-Whitney U test and Fisher's exact test were used to examine the significance of the difference in the laboratory parameters of the CHC patients with or without anti-CD69 autoAbs. p values of less than 0.05 were considered to be statistically significant. A multiple logistic regression analysis was used to elucidate independent predictive factors for the sustained response to IFN treatment.

Results

Reactivity of the Sera of Patients with Various Liver Diseases to Recombinant CD69 Protein

We investigated whether autoAbs to CD69 exist in sera of patients with various liver diseases by ELISA using the full-length recombinant CD69 molecule (fig. 1). As a result, IgG-type anti-CD69 autoAbs were detected in 24 out of the 61 patients with CHC (38.7%), in 6 out of the 47 patients with CHB (12.8%) and in 3 out of the 19 patients with AIH (15.8%) (fig. 2). On the other hand, autoAbs were detected in only 0–3.2% of the age- and sex-matched healthy donor groups for each category. Interestingly, none of the 19 asymptomatic HCV carriers tested showed positive reactivity to the CD69 molecule (fig. 2). Each positive serum sample was further confirmed to possess anti-CD69 autoAbs by Western blot analysis. Representative results are shown in figure 3.

Considering these data together, we found that autoAbs to CD69 existed in sera of patients with various types of chronic hepatitis. It is of interest that autoAbs to CD69 were much more common in patients with CHC than in those with CHB or AIH and were not detected in asymptomatic HCV carriers.

Epitope Mapping of CD69 Using Serum Samples from Patients with CHC

As described above, autoAbs to CD69 were much more common in patients with CHC than in those with CHB or AIH or in the asymptomatic HCV carriers. Thus, we recommend further studies concentrating on CHC. In our previous study, a predominant autoepitope was mapped at the C terminus of the CD69 molecule, using serum samples from patients with RA [24]. To under-

Table 1. Reactivity of the serum samples from patients with CHC to the four truncated CD69 proteins F1, F2, F3 and F3d by Western blotting

Sample No.	CD69			
	F1	F2	F3	F3d
6	+	+	+	+
8	+	+	+	+
16	±	+	+	+
19	+	+	+	+
21	−	+	+	±
25	−	+	+	+
27	±	±	±	+
31	+	+	+	±
34	+	+	+	+
41	±	+	+	±
43	+	+	+	+
44	+	+	+	+
46	+	+	+	+
47	+	+	+	+
49	+	+	+	+
51	+	+	+	±
52	+	+	+	±
59	+	+	+	+
60	−	+	+	±
61	±	+	+	−
64	+	+	+	+
68	+	+	+	+
70	±	+	+	−
71	+	+	±	+
Reactivity	21/24 (87.5)	24/24 (100)	24/24 (100)	22/24 (91.7)

The serum samples used were positive for the anti-CD69 autoantibody by ELISA.

Figures in parentheses represent percentages.

stand whether the mechanism for the production of anti-CD69 autoAbs in the patients with CHC was the same as that in RA patients or not, we investigated autoepitopes on the CD69 molecule, using the anti-CD69-positive serum samples from the patients with CHC. Specifically, we prepared four truncated fusion proteins of F1, F2, F3 and F3d [24] (fig. 1) and then investigated their antigenicity for the serum samples by Western blotting. F1, F2 and F3 covered the entire region of the CD69 molecules with overlaps. F3d is a small peptide region that carried the predominant autoepitope detected in our RA patients [24]. As a result, 19 out of the 24 serum samples (79%) reacted to all four fusion proteins F1, F2, F3 and F3d, and the remaining 5 serum samples reacted to three of the four regions (table 1). Representative results are shown in figure 4. This result was different from that obtained from the study on RA [24], in which most of the serum samples reacted solely to F3d.

Laboratory and Clinical Parameters of Patients with CHC with or without Anti-CD69 AutoAbs

To investigate pathophysiological roles of anti-CD69 autoAbs in CHC, we next compared clinical parameters between patients with anti-CD69 autoAbs and those without them. First, we compared biochemical parameters by blood examinations, including aspartate aminotransferase, alanine aminotransferase, albumin and prothrombin time, as shown in table 2. Also, we examined amounts of HCV RNA and the ratio of serogroup 1 to serogroup 2 of HCV (table 3). However, no significant difference was detected. Similarly, we compared the histological activity of the biopsied liver samples by the criteria of the histological activity index of Knodell et al. [29] between the two groups to elucidate the relation of anti-CD69 autoAbs with inflammation and fibrosis. However, no significant difference was observed (table 4).

Lastly, we compared the response to IFN-α therapy between the anti-CD69 autoAb-positive and -negative groups. Interestingly, 26 out of the 34 anti-CD69 autoAb-negative patients (76.5%) showed a sustained response (defined by negative HCV RNA 24 weeks after treatment) to IFN-α treatment (table 5). In contrast, only 5 out of the 20 anti-CD69 autoAb-positive patients (25%) met the criteria for a sustained response. Thus, the anti-CD69 autoAb could be a marker to predict response to IFN-α treatment. To evaluate whether the anti-CD69 autoAb was an independent factor for prediction, we applied multiple logistic regression analysis to the presence of anti-CD69 autoAbs as well as the amounts of HCV RNA and HCV serotypes tested here. As shown in table 6, although a high amount of HCV RNA (≥ 100 kcopies/ml) was the strongest predictive factor for the sustained response to IFN-α treatment ($p = 0.003$), the presence of the anti-CD69 autoAb was found to be another strong independent predictive factor ($p = 0.010$). HCV serotypes did not reach significance ($p = 0.086$). These data indicate that the anti-CD69 autoAb is an independent predictive factor for a sustained response to IFN-α treatment.

Laboratory and Clinical Parameters of Patients with CHB or AIH with or without Anti-CD69 AutoAbs

In addition to the analysis of CHC, we compared laboratory and clinical parameters in patients with CHB or AIH who did and did not demonstrate anti-CD69 au-

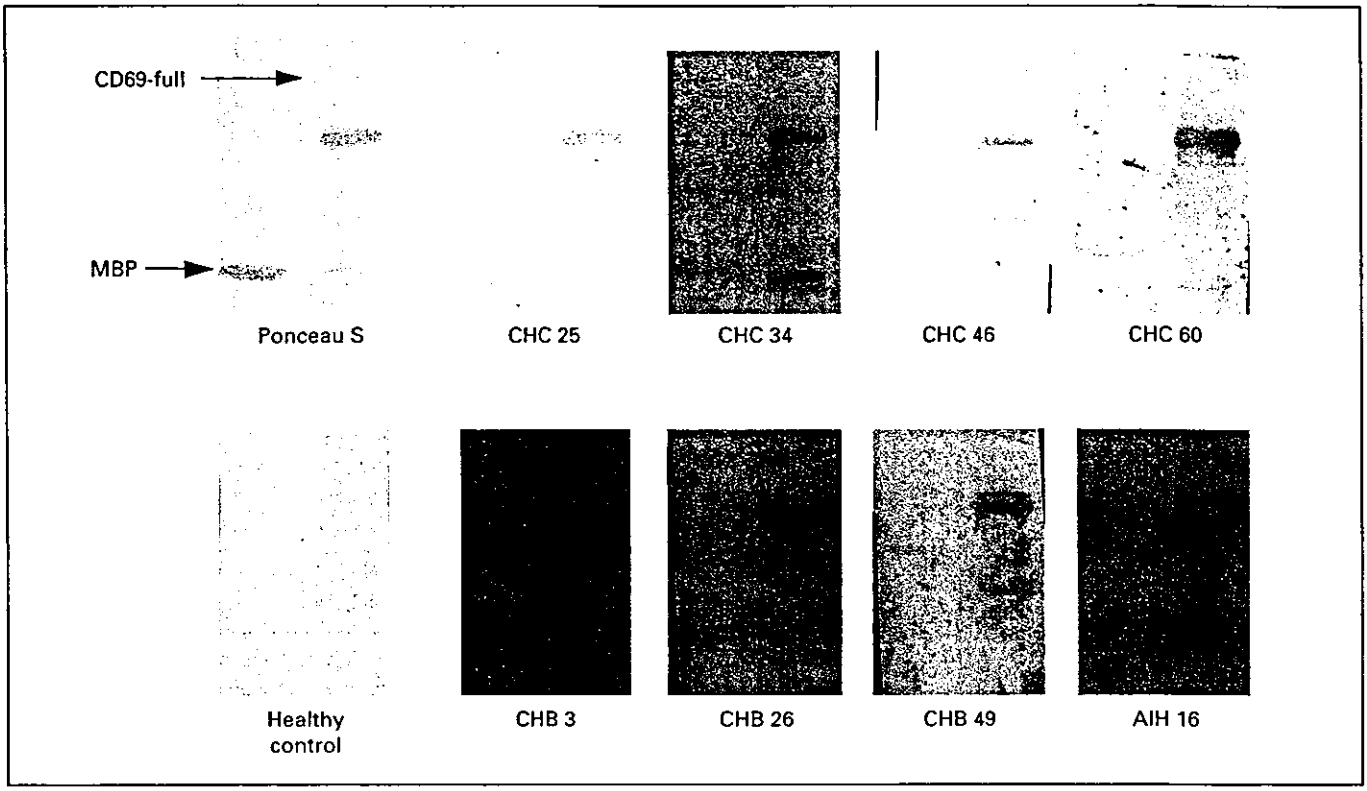

Fig. 4. Representative results of Western blotting of the truncated CD69 fusion proteins. The recombinant proteins CD69-full, F1, F2, F3 and F3d and MBP as a control were separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were reacted with serum samples diluted at 1:100 and then with horseradish peroxidase-labeled anti-human IgG antibodies. The bound autoAbs were visualized with diaminobenzidine.

toAbs. As a result, no significant difference was detected, as shown in tables 7 and 8, respectively.

Discussion

This study investigated the existence, production mechanisms and clinical significance of anti-CD69 autoAbs in patients with chronic hepatitis. Our results are as follows: 37.8% of the patients with CHC had anti-CD69 autoAbs, whereas only 12.8% of the patients with CHB and 15.8% of the patients with AIH had the autoAbs. None of the asymptomatic HCV carriers tested were positive for anti-CD69 autoAbs. Multiple epitopes on the CD69 molecule were recognized by most of the serum samples. The presence of anti-CD69 autoAbs had a reverse correlation with a sustained response to IFN-α therapy.

With regard to the first point, hepatitis C has been reported to be accompanied by various autoAbs like antinuclear antibodies, anti-smooth-muscle antibodies, antithyroid antibodies and anti-cardiolipin antibodies [18–21]. However, there have been no reports so far on antilymphocyte autoAbs. Therefore, we here investigated anti-CD69 autoAbs, one of the ALAs, which we first reported in patients with RA. As a result, we found that anti-CD69 autoAbs were detected most frequently in patients with CHC. Since more than 10% of the patients with CHB or AIH had anti-CD69 autoAbs, anti-CD69 autoAbs are not a specific marker for HCV infection. However, anti-CD69 autoAbs were 2–3 times as frequent as anti-nuclear antibodies and even more frequent than cryoglobulin, as shown in table 2. Thus, the anti-CD69 autoAb could be one of the representative autoimmune phenomena in CHC.

Second, the asymptomatic HCV carriers tested did not have anti-CD69 autoAbs, indicating that the presence of

Table 2. Comparison of blood biochemical parameters between anti-CD69 autoAb-positive and -negative patients with CHC

Parameter	AutoAbs to CD69		p
	positive (n = 24)	negative (n = 37)	
Aspartate aminotransferase, IU/l	80.6±47.2	78.7±52.0	NS
Alanine aminotransferase, IU/l	122.5±71.7	120.0±118.1	NS
Prothrombin time, %	88.1±12.2	93.1±11.0	NS
Albumin, g/dl	4.4±0.5	4.4±0.4	NS
Platelets, $\times 10^4/\mu l$	15.8±4.9	16.9±5.1	NS
ICG, R15%	12.5±10.2	12.4±8.7	NS
Rheumatoid factor, U/ml	11.7±6.9	425±2,035	NS
Erythrocyte sedimentation rate, mm/h	15.4±27	12.5±21	NS
C-reactive protein, mg/dl	<0.3	<0.3	NS
IgG, mg/dl	1,801±391	1,662±463	NS
IgA, mg/dl	222±124	240±122	NS
IgM, mg/dl	120±42	129±177	NS
TTT, U	9.7±9.8	7.6±4.8	NS
ZTT, U	17.7±4.8	15.5±6.2	NS
Positive ANA[1]	3/22 (13.6)	6/34 (17.6)	NS
Positive cryoglobulin[2]	6/15 (40	11/23 (48)	NS

p values were calculated using the Mann-Whitney U test. Figures in parentheses represent percentages. ICG = Indocianin green test; R15% = remnant rate after 15 min (%); TTT = thymol turbidity test; ZTT = zinc turbidity test; ANA = antinuclear antibody; NS = not significant.

[1] Data from 56 patients with CHC were available.

[2] Data from 38 patients with CHC were available.

Table 3. Comparison of the subtypes and concentrations of HCV between anti-CD69 autoAb-positive and -negative patients with CHC

HCV	Anti-CD69 autoAbs		p
	positive (n = 22)	negative (n = 33)	
Subtype[1]			
Serogroup 1	11 (50.0)	11 (33.3)	NS
Serogroup 2	11 (50.0)	22 (66.7)	NS
Serum HCV RNA, kcopies/ml[2]	399±411 (n = 14)	257±308 (n = 30)	NS

p values were calculated using the Mann-Whitney U test. Figures in parentheses represent percentages. NS = Not significant.

[1] Data from 55 patients with CHC were available.

[2] Data from 34 patients with CHC were available.

HCV itself is not linked to production of anti-CD69 autoAbs. Thereby, the anti-CD69 autoAb could be produced in the inflammatory process in the liver caused by HCV. Alternatively, the anti-CD69 autoAb could be one of the triggers to start the inflammatory process in the liver under CHV-infected conditions. Further studies will be needed to elucidate these mechanisms.

With regard to the third point, we previously reported that the anti-CD69 autoAb was detected in 38% of patients with RA. CHC and RA have several immunological disorders in common, such as frequent detection of rheumatoid factors and cryoglobulins [32]. Our study indicates that the anti-CD69 autoAb is another immunological disorder common to CHC and RA. In our previous

Table 4. Comparison of histological activity between anti-CD69 autoAb-positive and -negative patients with CHC

	HAI score	Anti-CD69 autoAbs		p
		positive (n = 24)	negative (n = 37)	
I.	Periportal ± bridging necrosis	1.75±1.11	2.08±1.38	NS
II.	Intralobular degeneration and focal necrosis	2.00±0.00	1.11±0.46	NS
III.	Portal inflammation	1.42±0.83	1.49±0.87	NS
IV.	Fibrosis	1.54±0.98	1.68±1.00	NS

p values were calculated using the Mann-Whitney U test. NS = Not significant; HAI = histological activity index.

Table 5. Difference in the responses to IFN-α therapy between anti-CD69 autoAb-positive and -negative patients with CHC

Response to IFN-α	Anti-CD69 autoAbs	
	positive (n = 20)	negative (n = 34)
Sustained response	5 (25.0)	26 (76.5)
No response	15 (75.0)	8 (23.5)

p = 0.0002 by Fisher's exact test. Figures in parentheses represent percentages.

Table 6. Variables related with resistance to IFN-α treatment by multiple logistic regression analysis

Variables	Odds ratio	95% CI	p
HCV RNA			
<100 kcopies/ml	1		
≥100 kcopies/ml	8.771	0.028–0.468	0.003
HCV serotype			
2	1		
1	3.831	0.052–1.308	0.086
Anti-CD69 autoAbs			
Negative	1		
Positive	8.850	0.028–0.457	0.010

Table 7. Comparison of blood biochemical parameters between anti-CD69 autoAb-positive and -negative patients with CHB

Parameters	Anti-CD69 autoAbs		p
	positive (n = 6)	negative (n = 41)	
Age, years	38.2±15.9	42.3±16.2	NS
Sex (M:F)	5:1	24:17	NS
Alanine aminotransferase, IU/l	312±283	193±172	NS
HBsAg positive	6	41	NS
HBeAg positive	4	34	NS
Anti-HBe positive	2	7	NS
HBV DNA, pg	615±197	833±199	NS
Histology of the liver			
Fibrosis[1]	2	1.5	NS
Overall histological activity[1]	1	2.3	NS

p values were calculated using the Mann-Whitney U test or Fisher's exact test. HBsAg = Hepatitis B s antigen; HBeAg = hepatitis B e antigen; HBV = hepatitis B virus; NS = not significant.
[1] Based on the METAVIR system.

Table 8. Comparison of blood biochemical parameters between anti-CD69 autoAb-positive and -negative patients with AIH

Parameters	Anti-CD69 autoAbs		p
	positive (n = 3)	negative (n = 16)	
Age, years	45.7±16.7	42.3±16.2	NS
Sex (M:F)	2:1	10:6	NS
Alanine aminotransferase, IU/l	279±283	810±899	NS
IgG, mg/dl	2,409±890	2,315±693	NS
Type of AIH			
Type I	3	14	NS
Type IV	0	2	NS
Histology of the liver			
Fibrosis[1]	1	1.4	NS
Overall histological activity[1]	1.7	2.3	NS

p values were calculated using the Mann-Whitney U test or Fisher's exact test. NS = Not significant.
[1] Based on the METAVIR system.

study [24], we found that most of the anti-CD69-positive RA serum samples recognized only one epitope nearly exclusively. The amino acid sequence of the predominant epitope is homologous to a part of low density lipoprotein receptor-related protein 2 (LRP-2), and, in fact, autoAbs to the predominant epitope of CD69 cross-reacted with the homologous region of LRP-2 [24]. Thus, we speculated that the anti-CD69 autoAb could belong to the anti-LRP-2 autoAbs. To investigate whether this scenario can be applied to anti-CD69 autoAbs in CHC, we mapped autoepitopes on CD69 using the serum samples of the tested CHC patients. As shown in table 3 and figure 4, the CHC serum samples mostly recognized all four fragments of CD69. This recognition of multiple epitopes indicates an antigen-driven immune response, not cross-reactive recognition. Thus, the mechanisms for the production of anti-CD69 autoAbs would be different between CHC and RA.

The last finding that the presence of anti-CD69 autoAbs had a significant correlation with resistance to IFN-α treatment in CHC is of clinical importance. Further, multiple logistic regression analysis revealed that the anti-CD69 autoAb was an independent predictive factor for a sustained response to treatment. Diagnostically, previous studies elucidated that the response to IFN-α treatment was influenced by several factors like viral genotypes, amounts of the viral RNA, the amino acid sequence of the IFN sensitivity-determining region and the histological grading [33–37]. However, there remain some patients whose response to IFN-α therapy cannot be predicted by the proposed predictive factors. Thus, the presence or absence of anti-CD69 autoAbs, as reported here, may be a novel useful marker for the prediction of response to treatment.

The question of how the presence of anti-CD69 autoAbs is linked to resistance to IFN-α therapy remains to be answered. Here, we could not detect substantial binding of anti-CD69 autoAbs to native CD69 on lymphocytes from CHC patients (data not shown). On this point, we previously reported the binding of anti-CD69 autoAbs to native CD69 on lymphocytes from RA patients; however, the binding was very weak [24]. Combining these data, autoAbs to recombinant CD69 may not bind substantially to natural CD69 on live lymphocytes. Therefore, although the anti-CD69 autoAb is a good marker, it would not have a direct effect on the resistance to IFN-α therapy, as is the case with the anti-Sm autoAb, which is a specific marker for systemic lupus erythematosus, but not has been shown to play a pathological role in the disease. Alternatively, we here detected autoAbs to recombinant CD69 molecules, which would not form conformational epitopes. AutoAbs to conformational epitopes, expressed only on native CD69, may have some pathological role. Further studies will be needed.

In conclusion, we have shown that the anti-CD69 autoAb is detected frequently in patients with CHC and that the presence of these autoAbs is linked to a negative response to IFN-α treatment. Further studies on CD69 autoAbs would provide us with clues to elucidate the inflammatory process of CHC and with diagnostic benefit in being able to predict responses to IFN-α therapy.

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Symptomatic therapies for prevention of hepatocellular carcinoma developing in chronic hepatitis C

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Ever since interferon (IFN) was found effective in clearing hepatitis C virus (HCV) from the circulation, it has become the mainstay for the treatment of chronic hepatitis C. In early clinical trials with IFN monotherapy, complete response with normalization of liver enzymes and clearance of detectable HCV RNA from serum, however, was achieved in only 5-30% of patients with chronic hepatitis C who received it. Response rates have been improved to 30-40% by long-term therapy with IFN alone or combined IFN and ribavirin. Development of pegylated IFN with a longer half-life and fewer side effects, especially when combined with ribavirin, is expected to increase the response rate to higher than 50%. Even with such an increase in response rate, however, attending physicians are left with the remaining ~50% of patients with chronic hepatitis C who fail to respond to the combined therapy and who continue to have HCV in the circulation.

Chronic hepatitis C in nonresponders can evolve to liver cirrhosis and eventually into hepatocellular carcinoma. Remarkable success with IFN and combined therapies averted, however, the general attention from nonresponders. It has become a pressing concern how to treat nonresponders to retard the progression of their liver disease, thereby preventing hepatocellular carcinoma. Fortunately, several lines of evidence have pointed to the possibility that the development of hepatocellular carcinoma can be retarded or aborted by suppressing necroinflammatory processes in the liver and keeping normal or low transaminase levels for many years. By so doing, the appearance of hepatocellular carcinoma would be postponed, hopefully, beyond the life expectancy of patients calculated as if they had no liver disease. Drugs for suppressing inflammation in the liver and lowering aminotransferase levels for symptomatic therapy of patients with chronic hepatitis C are reviewed, with a discussion of some clinical examples of their application.

Frontiers in Viral Hepatitis Ed. by RF Schinazi, J-P Sommadossi and CM Rice. 515 — 530

Introduction

In Japan, almost all patients who developed elevated levels of alanine aminotransaminase (ALT) in serum following a diagnosis of non-A, non-B hepatitis post-blood transfusion, have turned out to be infected with hepatitis C virus (HCV) by serological and molecular biological tests after HCV was discovered. Because the pathology in the liver does not progress rapidly, and remains mild even in patients with posttransfusion hepatitis having persistently elevated ALT levels for as long as 20 years, it may appear as if they have a favorable outcome. In individuals who have been infected with HCV for more than 20 years, however, liver pathology continues to progress. As time goes by, histology deteriorates from chronic hepatitis through liver cirrhosis (LC) to eventual hepatocellular carcinoma (HCC) at constant rates. Hence, posttransfusion HCV infection gradually lapses into serious morbidity with a poor prognosis.

HCV infection in Japan primarily spread in the community through illicit intravenous injection with methamphetamine just after the end of the World War II. A secondary transmission of HCV occurred by transfusion of blood units donated by drug addicts who were infected. Thirdly and probably most significantly, the pool of HCV increased in size through medical and paramedical practices with the use of insufficiently sterilized needles and syringes. Such a stepwise spread of HCV has resulted in more than two million people who are infected in Japan today [1].

As the most dreadful sequel of HCV infection in the past, the incidence of HCC has kept increasing yearly since 1975. As we reported earlier, the causality rate in Japan due to HCC gradually increased from 10 per 100,000 in 1975 to 35 per 100,000 in 1999. In addition, deaths due to liver cirrhosis number 10-20 per 100,000 yearly, and this has stayed rather constant [2].

This background obligates hepatologists in Japan to prevent a rapid increase in mortality due to HCV-associated chronic liver disease. Fortunately, the treatment of chronic hepatitis C patients with interferon (IFN) can reduce the development of HCC by half, as we reported previously [2]. In addition, we have found that IFN decreases the development of HCC not only in the patients with chronic hepatitis C who respond by clearing HCV RNA from the circulation (virological responders), but also in those in whom ALT levels are normalized or suppressed (complete or partial biochemical responders), despite the continuation of HCV infection after IFN. Our observations have been corroborated by subsequent reports [3, 4].

A six-month IFN monotherapy with a high initial dose, which has been given in Japan, induces sustained virological response in 30% of the recipients, of whom 70% were infected with HCV genotype 1b [2]. The response rate is similar to those achieved by a six-month combined therapy with IFN and ribavirin in many other countries. Large-scale clinical trials with combined IFN and ribavirin have been completed in the United States and Europe [5, 6]; the recipients are being followed for long-term outcome, with hopes for achieving great success. With the most optimistic projections, however, the rate for sustained virological response would not exceed 50% of the combined IFN and ribavirin recipients.

The use of pegylated IFN combined with ribavirin is presently under evaluation, which may further increase the response rate. Even with this regimen, however,

approximately 40% of the recipients are not expected to clear HCV from the circulation and will continue to have progressive liver disease. Accordingly, clinical hepatologists are burdened with nonresponders to the optimal therapeutic regimen of combined IFN and ribavirin, as well as the patients with chronic hepatitis C who can not receive it for various reasons. Hence, of utmost concern to attending physicians is how to decrease morbidity and mortality in these unfortunate groups of patients.

Symptomatic therapies for chronic HCV-associated liver diseases

In general, treatment of patients with chronic disease has been conducted with medications that have long-lasting, alleviating effects on symptoms, which has been the measure of their long-term efficacy (for example, drugs for normalizing hyperlipidemia in coronary heart disease, reduction of blood glucose levels in diabetes mellitus and reduction of blood pressure in hypertension). Symptomatic therapies with these drugs have been evaluated for their capacity to improve the prognosis. As to the results, they all have been found efficient in decreasing the morbidity and mortality associated with the respective diseases in the long run.

In the case of HCV-associated chronic liver diseases, some symptomatic clues have surfaced during long-term follow-up of patients. In patients who have been followed for a long time with checkups at regular intervals, those with low mean ALT levels did better, with reduced rates of progression to LC or HCC. Arase et al. [7] found in their clinical trial with a symptomatic therapy that HCC developed less often in the patients in whom ALT levels had been lowered artificially, and there was amelioration of inflammatory process in the liver. Their observations suggest, also, that lowered mean ALT levels during a long period would accompany a reduced rate of progression, from chronic hepatitis C to LC, and a decreased incidence of hepatic failure.

It has to be pointed out that medications for symptomatic therapy of chronic hepatitis have not been licensed. This rejection is based on the failure to achieve histological improvements during a short period spanning only one to two years, despite the fact that symptomatic drugs do lower ALT levels and, therefore, are thought to suppress necroinflammatory processes in the liver. The authorities may have been too hasty with their decisions on this issue.

Let's look at chronic hepatitis B, as an example. Individuals persistently infected with HBV who seroconvert to HBeAg (anti-HBe) either spontaneously or through treatment, and in whom hepatitis ameliorates, rarely show histological improvement, especially in the stage of hepatic fibrosis, within a short period of only 1-2 years. They still harbor HBV, and as a result probably continue to have slight to moderate inflammation in the liver.

This is actually the case with chronic hepatitis C. The patients with chronic hepatitis C who clear HCV from the circulation improve the histopathology in the liver during a follow-up of one to two years. Those who achieve sustained biochemical response with continuously normal ALT levels, but fail to respond virologically by clearing HCV RNA from serum, however, do not achieve improvement in hepatic fibrosis within a matter of one to two years [8], probably because slight inflammation goes on in the

liver; it takes five to 10 years for these patients with sustained biochemical response to have their liver histology improved. Fibrosis may not be lessened in degree for these patients; its rate of progression may be halted, however. Nonetheless, sustained normal or lowered ALT levels in serum, reflecting suppressed inflammatory processes in the liver, could prevent the evolution of chronic hepatitis to LC, and eventually into HCC, or retard this process, at least. Should this be achieved, mortality due to hepatic failure or HCC as sequelae of LC would be reduced substantially.

Hence, although killing and eradicating HCV is the principal goal in treatment of chronic hepatitis C, normalizing or lowering ALT levels accompanied by suppression of hepatic inflammation should not be taken lightly, especially in patients who do not respond to IFN virologically by clearing HCV RNA from serum. From this viewpoint, symptomatic treatment of chronic hepatitis C at present would deserve much more attention than ever.

1. Reduction of iron load

Di Biceglie et al. [9] reported that in chronic hepatitis, of type C in particular, serum levels of iron, ferritin and saturation of transferrin are increased frequently, while few cases exhibit increased iron in the liver. Their observations led them to suggest that liver injuries would be responsible for increased iron and ferritin levels in serum. Takikawa et al., however, found an iron load in the liver higher in chronic hepatitis C than B, and on that basis, have implicated hepatic iron deposition in elevated serum ALT levels in chronic hepatitis C. They went on to report a correlation between serum levels of ferritin and ALT in male donors positive for anti-HCV [10]. On the premise that iron overload would be involved in liver injuries associated with HCV infection, Hayashi et al. [11] evaluated the effect of maintaining ferritin levels $\leq$10 ng/ml by weekly or biweekly phlebotomy on serum ALT. The mean ALT decreased from pretreatment levels of 152 $\pm$ 49 to 55 $\pm$ 32 IU/L after therapeutic phlebotomy, with five of ten patients normalizing serum ALT levels. They found phlebotomy more efficient in the patients with higher activity of hepatitis [12].

Recently, the effect of increasing iron load on hepatitis was examined in chimpanzees by Bassett et al. [13] Chimpanzees infected with HCV, when they were placed on a high iron load, accumulated more iron in the liver than noninfected controls, accompanied by aggravation in histological pictures of chronic hepatitis. Thus, they have validated phlebotomy as a means of decreasing iron load and improving liver pathology in chronic hepatitis C.

Methods for reducing iron load are not restricted to phlebotomy, per se. An iron-deficient diet, as well as deferoxamine, a chelator of iron, are capable of reducing serum ALT levels in patients with chronic hepatitis. Kaltwasser et al. [14] gave black tea to patients with genetic hemochromatosis. Intriguingly, they observed that tannate in black tea reduced the absorption of iron, thereby reducing iron load in the liver. Their experience may be extended to patients with chronic hepatitis C for the purpose of reducing iron load.

Despite strong evidence for correlation between hepatic iron load and serum ALT levels, the mechanism is not fully explained. Farinati et al. [15], Sergent et al. [16] and

Weiss et al. [17] have tackled this issue to shed light on how iron disturbs hepatocytes and, conversely, why iron-reducing therapies work on chronic hepatitis. The present state of knowledge on these issues is summarized in the review by Bonkovsky et al. [18], as follows.

It is generally accepted that iron increases the formation of hydroxyl radicals and other highly reactive oxidizing molecules in biological systems. Secondary reactions and indirect effects lead to lipid peroxidation, oxidative damage to proteins and nucleic acids, and to a net increase in collagen and ground substance formation. In iron-loaded livers, such changes produce defects in organelle function (lysosomes, mitochondria, endoplasmic reticulum) and chronic deposition of scar tissue, and eventually hepatocellular carcinoma.

In addition, iron would have to be evaluated for the capacity to influence viral replication and mutagenesis, as well as for its direct or indirect effects on immune responses of the host.

Another practical aspect of an iron-reducing strategy relates to IFN therapy on chronic hepatitis C. van Thiel et al. [19] reported finding lower iron contents in the liver of responders to IFN. Their observations have been reproduced in studies that followed [20, 21], and point to the possible influence of iron reduction on increasing response rates to IFN. Some misunderstandings of 'response to IFN' may mar the judgement on the effect of iron reduction, however, which needs to be taken into consideration.

We have repeatedly discussed criteria for defining the efficacy of IFN on chronic hepatitis C [8, 22]. Our criteria have not achieved general consensus, mainly because the concept of 'response' differs widely depending on the doctors who use it, which has created much confusion. In the early days, when liver function tests were the only means of diagnosing non-A, non-B hepatitis, the response was judged by normalization of ALT levels during or at the completion of IFN therapy. Discovery of HCV has made it possible to detect HCV RNA in serum, and now it is increasingly used for evaluating the response to IFN therapy. Hence, 'response to IFN' has dual connotations at the present time, one of which is biochemical (normalization of serum ALT), while the other is virological (loss of HCV RNA from serum). These two distinct kinds of responses can create misjudgement in evaluating the efficacy of IFN therapy, because they do not associate.

Patients with low pretreatment values of serum iron and ferritin have lower baseline ALT levels than those with high values. Hence, they are predisposed already to normalizing ALT levels after receiving IFN, which does not necessarily correlate with the loss of HCV RNA by IFN. Likewise, the implied effects of iron reduction prior to IFN therapy, to increase efficacy, need to be taken with caution; they can simply reflect ramifications of reduced iron load, as such, that may have been erroneously counted in for overestimating the true 'response' to IFN. Despite these serious reservations, the overall effect of iron reduction has been estimated impartially in previous studies, regardless of the judgement of 'response,' either by normalized ALT or loss of HCV RNA, which has produced deep confusion. To help answer this question, a randomized placebo-controlled clinical trial was conducted to evaluate the net effect of iron reduction on increasing the rate of sustained response (SR) in IFN therapy [23].