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Abstract

It has been documented that the serum complement activities measured by hemolytic assay (CH50) are decreased after storage of sera at a low temperature in some patients with chronic hepatitis C. However, the mechanism of this phenomenon has not been identified yet. Here, we tried to elucidate factors involved in the cold activation of complement (CAC). To clarify what pathway is activated in CAC, we measured complement cleavage products after cold storage of sera. C4d increased significantly after 12 h-storage at cold temperatures in 5 CAC (+) sera compared with 5 CAC (-) ($P < 0.01$) and 3 control sera ($P < 0.05$), while Bb did not increase in any of the groups. In order to determine whether IgG or IgG complex is necessary for CAC, 8 CAC (+) sera were incubated with Protein G Sepharose gel beads, and all of them retained hemolytic activities to some extent after cold storage. Column chromatography through Superose 6HR of CAC-positive serum identified the fractions containing molecules that induced CAC in normal serum, which were depleted by treatment with protein G Sepharose. In conclusion, CAC in hepatitis C seems to occur via a classical or lectin pathway, and the IgG complex produced in hepatitis C virus infection may be an important factor in inducing CAC, a common extrahepatic manifestation of hepatitis C.

KEYWORDS: hepatitis C virus, chronic hepatitis, complement activation

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Original Article

Cold Activation of Serum Complement in Patients with Chronic Hepatitis C: Study on Activating Pathway and Involvement of IgG

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It has been documented that the serum complement activities measured by hemolytic assay (CH50) are decreased after storage of sera at a low temperature in some patients with chronic hepatitis C. However, the mechanism of this phenomenon has not been identified yet. Here, we tried to elucidate factors involved in the cold activation of complement (CAC). To clarify what pathway is activated in CAC, we measured complement cleavage products after cold storage of sera. C4d increased significantly after 12 h-storage at cold temperatures in 5 CAC (+) sera compared with 5 CAC (−) ($P < 0.01$) and 3 control sera ($P < 0.05$), while Bb did not increase in any of the groups. In order to determine whether IgG or IgG complex is necessary for CAC, 8 CAC (+) sera were incubated with Protein G Sepharose gel beads, and all of them retained hemolytic activities to some extent after cold storage. Column chromatography through Superose 6HR of CAC-positive serum identified the fractions containing molecules that induced CAC in normal serum, which were depleted by treatment with protein G Sepharose. In conclusion, CAC in hepatitis C seems to occur via a classical or lectin pathway, and the IgG complex produced in hepatitis C virus infection may be an important factor in inducing CAC, a common extrahepatic manifestation of hepatitis C.

C old activation of complement (CAC) is an *in vitro* phenomenon in which the serum complement activity measured by hemolytic assay (CH50) decreases after storage of sera at a low temperature in some patients whose serum or plasma has a normal hemolytic activity when tested immediately after separation [1-3]. This phenomenon was initially reported concerning sera from patients with chronic non-A, non-B liver diseases [4, 5]. And recently, it has been clarified that CAC is closely related to hepatitis C virus (HCV) infection [6, 7]. In our experience, 42% of 202 patients with chronic hepatitis C showed low CH50 in spite of a normal range of serum C3 and C4 [8]. Previous investigations showed that there were no differences in clinical or viral backgrounds between CAC (+) and CAC (−) patients [7]. In a previous report on chronic hepatitis C patients, only rheumatoid factor showed a significant difference between patients with normal and very low CH50 [8].

To elucidate the mechanism of CAC in chronic HCV infection, the activation pathway of complements and the role of immunoglobulins for CAC were assessed in this study.

Materials and Methods

Patients. Thirteen patients with chronic hepatitis C and 3 healthy subjects were enrolled in this study. In hepatitis patients, 8 were positive for CAC and 5 were negative (Table 1).

Sera. Sera from patients were separated after clotting blood at 37 °C for 2 h, then immediately stored at −80 °C until use.

Determination of CH50. CH50 was determined by using modified Mayer's method (new one-point CH50 kit; Japan Lyophilization Laboratory, Tokyo, Japan) according to the manufacturer's instructions. In brief, $10 \mu l$ of serum were added to 3 ml of gelatin veronal buffer (GVB) and $50 \mu l$ of suspension of sheep red blood cells sensitized with rabbit hemolysin and incubated at 37 °C for 60 min. The lysate was centrifuged at 2,000 rpm for 5 min at 4 °C, and the optical density (OD) of the supernatant was measured at 541 nm. In this study, OD_{541} of lysate was used as the arbitrary units for complement activity. CAC was defined as positive when serum had a normal CH50 (30–40 U/ml) just after its separation and less than 12.5 U/ml after storage at 4 °C for 24 h. On the other hand, CAC was defined as negative when serum with normal CH50 just after separation remained within the normal range of CH50 after storage at 4 °C for 24 h. CAC was not assessed for serum with < 30 U/ml of CH50 just after separation.

Measurement of C4d and Bb. C4d and Bb, cleavage products of C4b and factor B, in serum stored at 4 °C, were measured by using an ELISA kit (QUIDEL, San Diego, CA, USA).

Protein G Sepharose immunoabsorption. To delete IgG from serum, $200 \mu l$ of serum was incubated with $100 \mu l$ of packed Protein G Sepharose 4 Fast Flow beads (Pharmacia LKB Biotechnology, Uppsala, Sweden) at 37 °C for 5 min and was spun down to remove the supernatant. Anti-glycophorin Sepharose beads, kindly provided by Dr. Teizo Fujita, Fukushima Prefectural Medical College, were used as the control. Elution from protein G Sepharose was performed by use of a

Glycine buffer, pH 2.7 after vigorous washing with phosphate buffered saline (PBS).

Fast protein liquid chromatography (FPLC). Two hundred microliters of serum were fractionated through Superose 6 HR 10/30 (Pharmacia LKB Biotechnology) with PBS at a flow rate of 0.5 ml/ min at room temperature. Each 1.5 ml fraction was collected, and 1 ml aliquot of each fraction was mixed with $20 \mu l$ of normal serum, stored at 4 °C for 24 h, and hemolytic activity was determined. The IgG and IgM of each fraction was determined by the immunodiffusion method with anti-human IgG or anti-human IgM rabbit serum (DAKO, Glostrup, Denmark).

Determination of HCV-RNA and anti-HCV antibody. HCV-RNA was determined by reverse transcription-polymerase chain reaction as reported previously [9, 10]. Anti-HCV core antibody was determined at a commercial laboratory (Shionogi Biomedical Laboratories, Tokyo, Japan) by means of immunoradiometric assay.

Statistical analysis. The statistical comparisons were evaluated by Chi-square test, Mann-Whitney U test and Wilcoxon signed rank sum test.

Results

Clinical backgrounds of patients (Table 1). Between CAC (+) (patients 1–8) and CAC (−) (patients 9–13) patients, there were no statistically significant differences in age, sex, alanine aminotransferase level, or concentration of serum γ-globulin, C3, and C4. Also, HCV markers such as anti-HCV core antibody, HCV genotype, and viral load were not different between the 2 groups.

Complement cleavage products from serum stored at 4 °C. C4d and Bb, cleavage products from C4b and factor B, were measured before, 12 h after and 24 h after storage at 4 °C (Fig. 1). Concentrations of C4d in 5 CAC (+) sera (Table 1, patients 1–5) increased significantly after storage at 4 °C for 12 h ($P < 0.05$) and for 24 h ($P < 0.05$). They were significantly higher than those of 5 CAC (−) sera (Table 1, patients 9–13) and 3 normal controls after 12 h ($P < 0.01$ and $P < 0.05$, respectively) and after 24 h ($P < 0.05$ and $P < 0.05$, respectively). Concentrations of Bb did not change after storage at 4 °C in any sera.

**The effect of IgG depletion from serum on CAC phenomenon.** All of 8 samples of CAC (+)

Table I   Clinical backgrounds of patients

Patient	Age / Sex	CH50 (U/ml) incubation at 4 °C		ALT (IU/l)	γ-Globulin (g/dl)	C3 (mg/dl)	C4 (mg/dl)	Rheumatoid Factor (IU/ml)	Anti-HCV core antibody (U)	HCV genotype	HCV-RNA (Meq/ml)
		Before	After								
Cold activation positive											
1	38/F	33.6	< 12.5	17	1.4	52.7	19.2	23.2	280	1b	6.4
2	63/F	44.0	< 12.5	53	2.3	99.2	31.5	55.9	N.D.	1b	6.4
3	32/F	32.1	< 12.5	16	1.1	59.6	19.3	< 17.7	170	2b	0.84
4	52/M	32.6	< 12.5	118	1.9	69.8	22.2	20.1	430	1b	< 0.5
5	72/F	36.8	< 12.5	43	1.6	64.5	21.8	18.1	N.D.	1b	N.D.
6	45/F	36.3	< 12.5	33	1.3	N.D.	N.D.	N.D.	240	1b	0.77
7	55/M	34.1	< 12.5	129	1.6	68.1	26.1	34.5	N.D.	1b	15
8	36/F	31.6	< 12.5	53	1.9	71.7	14.3	< 17.0	N.D.	1b	6.8
Cold activation negative											
9	62/M	38.6	37.3	59	1.4	74.0	25.3	< 17.3	N.D.	1b	23
10	63/M	39.1	38.1	28	1.1	67.9	21.4	< 16.7	66	1b	N.D.
11	58/F	35.0	33.6	41	2.1	66.9	19.4	245.1	67	1b	16
12	64/M	35.9	35.9	56	0.9	53.9	23.0	< 17.7	430	1b	0.80
13	35/M	34.5	34.1	38	0.7	69.0	26.9	< 16.5	200	2a	< 0.5

ALT, Alanine aminotransferase; N.D., not determined.
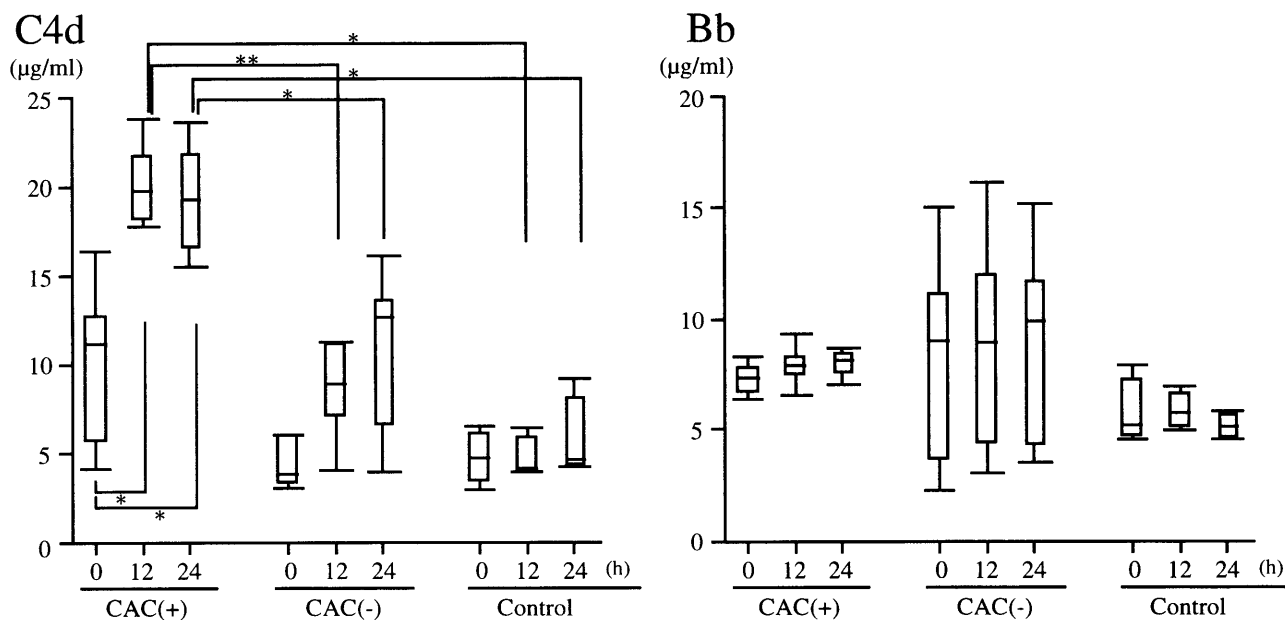
Fig. I   Change of concentration of C4d and Bb in sera during storage at 4 °C. Sera from chronic hepatitis C patients with cold activation of complement (CAC (+); n = 5), without CAC (CAC (−); n = 5) and from healthy controls (n = 3) were stored at 4 °C for 24 h. Concentrations of C4d and Bb were measured by methods described in text. Values are shown in box graph expressing range: bar in the box, median; lower and upper side of box, 25 and 75 percentile; lower and upper bar, minimum and maximum. *$P < 0.05$; **$P < 0.01$. Normal values were as follows: C4d 1.2–8.0 μg/ml; Bb 1.0–7.3 μg/ml.

serum (Table 1, patients 1–8) were incubated with protein G Sepharose beads to deplete IgG and hemolytic activity was measured to determine complement activation. All of the samples preserved hemolytic activity to some extent after storage at 4 °C and after treatment with protein G Sepharose, while severe cold activation occurred in sera treated with anti-glycophorin Sepharose (Fig. 2).

HCV-RNA was detected in 3 out of 5 eluates from protein G Sepharose beads.

Column chromatography. CAC-positive serum from patient 3 without and with IgG depletion by protein G Sepharose treatment was fractionated through FPLC into 20 fractions (Fig. 3). IgG concentrations were 1,228 mg/dl and 372 mg/dl, respectively. IgM was eluted at fraction 7, and IgG was eluted at fraction 10 through 12. Without IgG depletion, the addition of fraction 7 to 9 to normal serum induced CAC (Fig. 3A). The same fractions from IgG-depleted serum did not induce CAC (Fig. 3B).

Discussion

Cold activation of complement refers to the phenomenon by which the complement system is activated at low temperatures. This phenomenon after storage at 4 °C is recognized by a decrease of CH50 titer of serum that is normal just after bleeding. Kondo and Inai reported that this phenomenon was observed with a high frequency in sera from patients with chronic liver diseases [4, 5]. Tsuji suggested that this complement activation occurred only in sera from patients with non-A, non-B hepatitis, but not with hepatitis B [11].

In 1989, hepatitis C virus (HCV) was identified as a causative agent for non-A, non-B hepatitis [12, 13]. Ueda and Itoh indicated that cold activation of complement is closely related to HCV infection [6, 7]. When CAC (+) hepatitis C patients responded completely via interferons during treatment and eradicated the circulating virus, their CAC turned out to be negative [8, 14]. This indicates that CAC is related to the presence of HCV. However, the mechanism of this phenomenon has not been identified yet.

In this study, when the serum was CAC-positive, the concentration of C4d increased during storage at a cold temperature. On the other hand, it did not increase in CAC-negative serum (Fig. 1). However, the concentration of Bb did not increase in either CAC-positive or -negative groups. Three pathways for the activation cascade of the complement system have been identified, namely, classical, alternative, and lectin pathways [15].

Protein G Sepharose

OD$_{541}$

Control
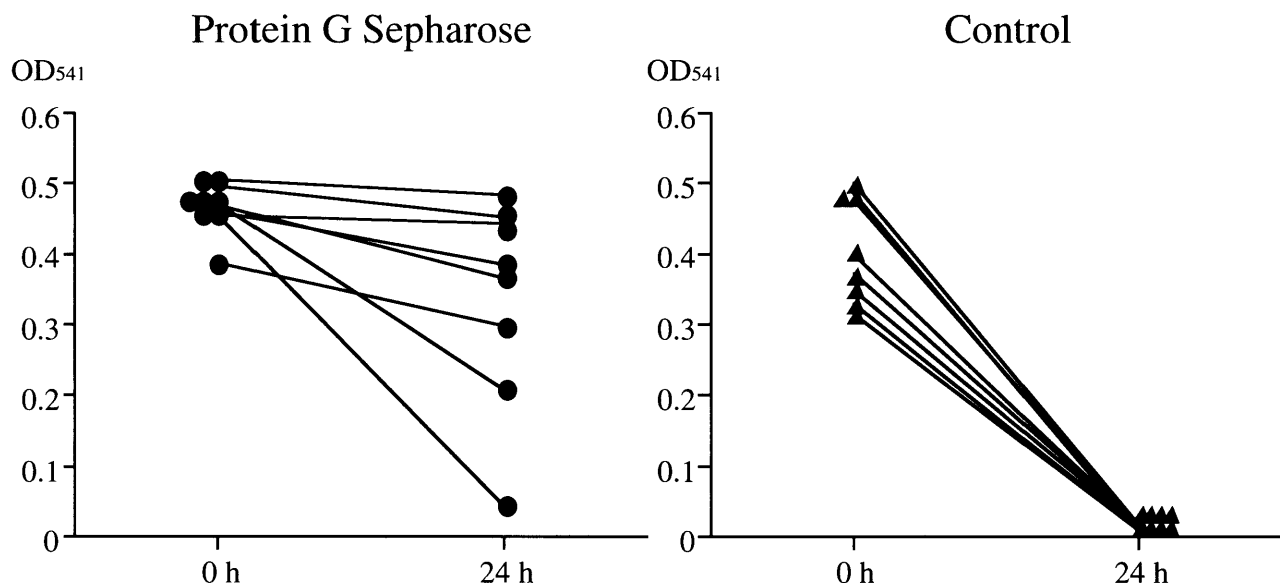
OD$_{541}$

Fig. 2   Hemolytic activity of CAC (+) sera after IgG depletion. After CAC (+) sera were treated with protein G Sepharose beads, hemolytic activity (OD$_{541}$) before and after storage at 4 °C for 24 h was measured by methods in text (●). Anti-glycophorin Sepharose beads were used as a control (▲).

(A)

(B)
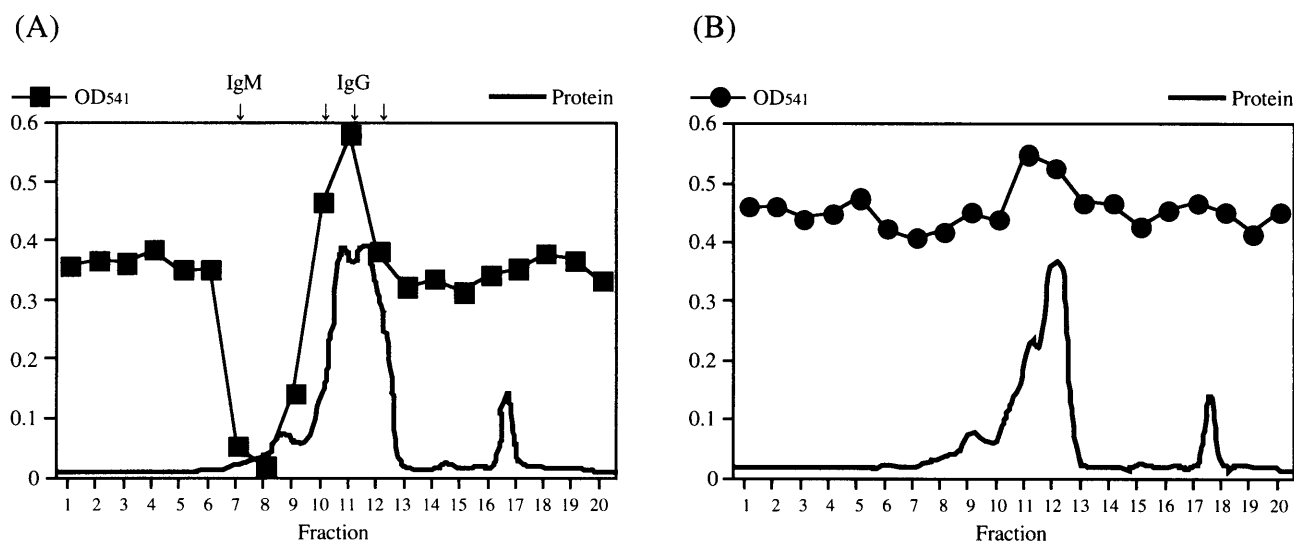
Fig. 3   Cold activation of complement by each fraction eluted through FPLC of original (panel **A**) and protein-G-treated (panel **B**) serum. CAC (+) serum (Table I, patient 3) was chromatographed through Superose 6 HR as described in text. Protein concentration was monitored by measuring OD_{280} (curved line). Hemolytic activity (OD_{541}) of normal serum mixed with each fraction after storage at 4 °C for 24 h was plotted (■, original serum; ●, protein-G-treated serum).

C4b is cleaved to C4d when the classical or lectin pathway is activated. On the other hand, factor B is degraded through an alternative pathway of complement activation. These 2 factors are cleaved exclusively in each pathway. So, these results indicated that CAC in hepatitis C occurred via a classical pathway or lectin pathway, not an alternative pathway. Kitamura showed that C4 and C2 in sera of CAC-positive patients with non-B hepatitis were activated and degraded after cold storage; however, activities of C3–9 were normal. So, he suggested that CAC occurred through a classical pathway [16], and this is consistent with our results.

To clarify the role of immunoglobulin in CAC, IgG fractions were depleted from CAC-positive serum by incubation with protein G Sepharose beads. IgG-depleted sera retained hemolytic activity after storage at 4 °C for 24 h (Fig. 2), and HCV-RNA was detected in 3 out of 5 eluates from protein G Sepharose beads. This suggested that molecules in serum bound to protein G Sepharose beads, which may include an IgG immune complex with HCV, play an important role in CAC. Although there was no difference of anti-HCV core antibody titer between CAC (+) and CAC (−) patients (Table 1), anti-envelope titers should be compared.

Mathews showed that the CAC phenomenon was reduced by adsorption with streptococcal protein G

Sepharose in one patient who had a low CH50 level and no liver disease [17]. Atkinson showed that the CAC phenomenon in systemic lupus erythematosus was reduced by partial IgG-depletion by ammonium sulfate precipitation and DEAE column chromatography [18]. These reports indicated that IgG not related to HCV infection might also be involved in CAC.

Fractions from CAC (+) serum by Superose 6HR chromatography were used to reconstitute the phenomenon in serum from healthy subjects. In this experiment, molecular weights in the No. 7–No. 9 fractions which induced CAC in normal serum were smaller than IgM and larger than IgG (approximately 150 kDa–900 kDa) (Fig. 3). The same fractions from protein-G-treated serum did not induce CAC. Factor (s) inducing CAC with a molecular weight of this range was/were present in serum from patients infected with HCV. Further characterization of these factors would clarify the mechanism of CAC.

Wei reported that CAC was induced by an immune complex including IgG or aggregated IgG or IgG-IgM complexes fractionated by sucrose density gradient ultracentrifugation of the eluates from the protein G Sepharose column of a CAC-positive patient [19]. Circulating immune complexes in acute and chronic liver diseases, as well as in autoimmune diseases, have been detected with great frequency and high titers [20, 21].

Tsai showed that 96.3% of sera had a high level of IgG-conglutinin, IgM-conglutinin, IgG-C1q, or IgM-C1q in a chronic hepatitis C patient [22].

The structure of human IgM rheumatoid factor bound to IgG Fc has recently been recognized, and this complex can cause inflammation of rheumatoid arthritis by activating a complement [23]. In our previous report on chronic hepatitis C patients, there was a difference in the titer of rheumatoid factor between patients with normal and very low CH50 [8]. Our FPLC study suggested that molecular weights of factors inducing CAC were smaller than IgM, so an IgM or IgM-IgG complex might play a less important role in CAC. It is suggested that some IgG complexes constructed under the existence of HCV may cause CAC.

In our preliminary study, CAC still occurred after depletion of mannose-binding lectin, one of the key initiating factors of the lectin pathway [15], from CAC (+) serum (data not shown). Although further studies are necessary, the lectin pathway may be less important for CAC.

In HCV infections, several extrahepatic manifestations are observed. Some of them are immunological abnormalities, such as an autoimmune phenomenon [24, 25], cryoglobulinemia [26, 27], and rheumatoid reaction [19]. It still remains unclear why HCV infection induces such abnormal immunological phenomena. CAC is one of such extrahepatic manifestations of HCV infection, and the elucidation of the pathogenesis of CAC could lead to a greater understanding of the immunological status in HCV infection.

In conclusion, the complement in sera from patients infected with HCV is activated *in vitro* at cold temperatures via a classical or lectin pathway, and the IgG complex may play an important role in CAC.

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