A Study of Erythrocyte C3b Receptors (E-CR1) in Patients with Acute Viral Hepatitis

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Abstract

Erythrocyte C3b receptors (E-CR1) were assayed by ELISA in 16 patients with acute viral hepatitis. In 13 patients with hepatitis type A, type B, or type non-A non-B, the E-CR1 level was significantly lower in the acute phase than in healthy controls, and recovered in the convalescent phase. The E-CR1 levels in 3 patients with non-A, non-B hepatitis remained below the mean level of the healthy controls. These findings suggest that the E-CR1 level fluctuates in acute viral hepatitis, and that it continues to be reduced in some patients with non-A non-B acute hepatitis.

Introduction

In 1953, Nelson found that antigen-antibody complexes adhere to primate erythrocytes via complement, and Nishioka et al. showed that this adhesion is due to binding of the third complement component to C3b receptors (CR1s) on the surface of human erythrocytes. In 1983, Cornacoff et al. showed that immune complexes (ICs) injected intravenously into baboons immediately bound to erythrocytes but that ICs bound to erythrocytes had been completely eliminated in the hepatic vein. From these reports, the erythrocyte-CR1 (E-CR1) is considered to be involved in the transport and elimination of ICs.

The presence of ICs has been reported also in acute viral hepatitis and various liver diseases. The presence of ICs is liver diseases is considered to be reflected by the E-CR1 level, but, to our knowledge, there has been only one report of E-CR1 assay in liver diseases, that by Kondo et al. In the present study, we serially determined the E-CR1 level in patients with acute viral hepatitis by ELISA and studied its clinical significance in this disease.

Materials and Methods

1. Subjects

The subjects were 16 patients with acute viral hepatitis who were admitted to our department or related hospitals between June 1989 and May 1990 and were available for follow-up studies and serial blood sampling. As shown in Table 1, they consisted of 6 patients with acute hepatitis type A (AHA) definitively diagnosed by the positive for IgM anti-HAV antibody, 3 patients with acute hepatitis type B (AHB) in whom HBs antigen was positive and IgM class anti-HBc antibody was increased and HBs antigen disappeared during the course, and 7 patients with non-A non-B acute hepatitis (AHNANB) in whom AHA and AHB, alcohol— or drug-induced hepatic injury, and primary infection with cytomegalovirus or Epstein-Barr
virus could be excluded. The subjects were 7 males and 9 females ranging in age from 17 to 69 years (40.2 ± 16.5 years, mean ± SD). The maximum sGPT level during the course of the disease was 1998.3 ± 2132.5 KU, the mean red cell count on admission was 491 × 10⁶/μl, and the mean hemoglobin level (Hb) was 14.6 g/dl.

Blood was collected from each patient every week from right after admission and general liver function tests were performed. E-CR1 was measured by the method described below. The period within 1 week after the onset was regarded as the time of onset, 2-4 weeks after the onset as the acute phase, and 5 or more weeks after the onset as the convalescent phase.

E-CR1 was measured in 8 healthy individuals (4 males and 4 females) as a control group. They were 24-34 years old (27.6 ± 4.1 years).

2. Measurement of E-CR1 by ELISA
1) Samples and measurement
Venous blood collected with citrate-phosphate-dextrose-adenine was stored at 4°C and measurements were made within 1 week.

2) Methods
E-CR1 was measured by the method of Thomsen et al.8) The blood samples were washed 3 times with phosphate-buffered saline (PBS, pH 7.4), and the erythrocytes were suspended in PBS at 5 × 10⁷ cells/ml. One hundred μl of 0.01% poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA) was transferred to a 96-well polystyrene flat bottomed plate (C.A. Greiner und Sohne, Nutingen, Germany), and incubated at 22°C for 30 minutes. The poly-L-lysine was discarded, the plate was washed 3 times with PBS supplemented with 0.05% Tween 20 (PBS-T), 100 μl of the above erythrocyte suspension was added (5 × 10⁶ cells/well) and the plate was allowed to stand at room temperature for 30 minutes. Then the solid-phase erythrocytes were obtained by gently adding 100 μl of 0.1% glutaraldehyde and allowing them to react at 22°C for 30 minutes. The solid-phase erythrocytes were washed 3 times with PBS-T, and the formation of an erythrocyte monolayer was confirmed by phase microscopy. Wells with poorly formed monolayers were not used.

Next, the solid-phase erythrocytes were incubated with 250 μl of PBS containing 1% BSA at 22°C for 60 minutes and washed 3 times with PBS-T. Anti-human CR1 mouse monoclonal antibody (MoAb; Dakopatts, Copenhagen, Denmark) diluted 100 times with PBS was added and allowed to react with the cells at 22°C for 60 minutes. The wells were washed 3 times with PBS-T and were allowed to react with 100 μl of a 100-fold PBS solution of ALP-labeled anti-mouse IgG rabbit immunoglobulin (Dakopatts) at 22°C for 60

Table 1 Patient profiles.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
<th>Age (years)</th>
<th>Sex (M:F)</th>
<th>Maximal level of sGPT (K.U)</th>
<th>RBC (×10⁶/μl)</th>
<th>Hb (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHA</td>
<td>6</td>
<td>36.5±14.4</td>
<td>3:3</td>
<td>3380.3±2819.1</td>
<td>502.3±64.9</td>
<td>14.7±1.5</td>
</tr>
<tr>
<td>AHB</td>
<td>3</td>
<td>33.7±13.3</td>
<td>2:1</td>
<td>1751.0±1090.9</td>
<td>485.3±70.8</td>
<td>14.7±1.9</td>
</tr>
<tr>
<td>AHNANB</td>
<td>7</td>
<td>46.1±17.3</td>
<td>2:5</td>
<td>919.7±495.4</td>
<td>482.5±46.0</td>
<td>14.5±1.6</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>40.2±16.5</td>
<td>7:9</td>
<td>1998.3±2132.5</td>
<td>491.0±60.1</td>
<td>14.6±1.6</td>
</tr>
</tbody>
</table>

AHA: acute hepatitis type A
AHB: acute hepatitis type B
AHNANB: acute hepatitis type non-A non-B

Values are Mean±SD
minutes. Then, they were washed 3 times with PBS-T and the color reaction was performed by treating the wells 200 μl of a 4 mg/ml disodium p-nitrophenyl phosphate solution (Sigma) at 37°C for 120 minutes. The reaction was stopped by transferring 150 μl of the reactants to a flat-bottomed plate, to each well of which 100 μl of 1N-NaOH, was added in advance and the absorbance at 405 nm was determined. Determinations were made in quadruplicate. Since this method employs anti-human CR1 MoAb as the primary antibody, the decrease in CR1 may be direct or indirect if ICs are bound to receptors or if autoantibody to E-CR1 is present.

The value of E-CR1 was expressed as follows. The erythrocyte sample from a healthy person in which the absorbance was the highest in ELISA was regarded as the standard control, and the percent of E-CR1 values in other samples as compared with this standard control was calculated by the formula CR1 level (%)=sample O.D./standard control O.D. × 100. The coefficient of variation of the absorbance of the standard control was less than 10%.

To determine the relationship between the number of solid-phase erythrocytes and the E-CR1 level, the following experiment was performed. Twofold serial dilutions of the erythrocyte suspension of the standard control were prepared. The erythrocyte suspension at each concentration was treated in its well to obtain the solid phase. To obtain the number of solid-phase erythrocytes, the solid-phase erythrocyte Hb concentration was obtained as the O.D. value at 405 nm. Simultaneously, the E-CR1 level for solid-phase erythrocytes was determined as the O.D. value using the same serial dilutions (Fig. 1A, 1B). When erythrocyte suspensions at concentrations between 0.078 × 10⁶/well and 0.625 × 10⁶/well were used to obtain the solid phase, the Hb concentration was related to the E-CR1 level. When the concentration of the

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**Fig. 1 A:** Relationship between the number of erythrocytes per well and hemoglobin concentration assayed by ELISA.

**Fig. 1 B:** Relationship between the number of erythrocytes per well and erythrocyte CR1 level assayed by ELISA.

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erythrocyte suspension was between $1.25 \times 10^6$/well and $5.0 \times 10^6$/well, both Hb concentration and the E-CR1 level reached a plateau, showing no significant changes in the O.D. value. These results suggest that the maximum number of solid-phase erythrocytes in each well is $1.25 \times 10^6$. Therefore, the erythrocyte suspension ($5 \times 10^6$) used in this study contained more than an adequate number of erythrocytes that would be changed to the solid phase in the well. These results also suggest that changes in the E-CR1 level can be compared among samples when the number of erythrocytes per well that change to the solid phase is constant.

3. Statistical analysis

Data were tested for significant differences by student’s t-test.

Results

1. E-CR1 level in healthy controls (Table 2)

In the 8 healthy individuals, the E-CR1 levels were distributed in a range of 56.3-100% (80.7 ± 13.9%).

2. Changes in the E-CR1 level in the course of hepatitis (Figs. 2 and 3)

Changes in the mean sGPT level and the E-CR1 level in the 6 AHA patients and the 3 AHB patients are shown in Fig. 2. The sCPT level showed a monophasic peak and the expected recovery thereafter in both
AHA and AHB patients. The E-CR1 level decreased to 49.7-68.9% (58.0 ± 6.2%) in the acute phase 2-4 weeks after the onset in AHA patients, but increased thereafter and reached 74.6-108% (84.3 ± 14.8%) in the convalescent phase 7-8 weeks after the onset. The E-CR1 level showed similar changes in AHB patients; it decreased to 30.5, 55.4, and 68.0% (mean 51.3%) in 3 patients 2-4 weeks after the onset but increased to 72.3, 112.8, and 117.0%, respectively (mean 100.7%) 6-9 weeks after the onset.

Fig. 3 shows changes in the E-CR1 level in all 7 AHNANB patients. Unlike AHA or AHB, there was no particular tendency, and the changes in 4 patients solid lines were similar to those in AHA and AHB patients. In one of these patients, the E-CR1 level increased after the onset and reached 118% after 5 weeks. In the other 3 of these patients, it temporarily decreased to 44.1, 69.2, and 75.3% (mean 61.9%) after 2-4 weeks but increased to 83.4, 85.1, and 98.4%, respectively (mean 89.0%) after 5-6 weeks. In the remaining 3 patients (broken lines), however, the E-CR1 level continued to be less than 70% except for a temporary increase to 75.6% in one of them.

3. Comparison of the E-CR1 levels in healthy controls and patients with hepatitis in various phases (Fig. 4)

The maximum and minimum E-CR1 levels were compared in the 8 healthy controls and 7 hepatitis patients (3 with AHA, 1 with AHB, and 3 with AHNANB) in whom the E-CR1 level, followed from immediately after the onset, decreased in the acute phase and increased in the convalescent phase. In the patients, the E-CR1 levels at the onset, in the acute phase, and the convalescent stage were 90.0 ± 15.6, 57.4 ± 15.8, and 84.6 ± 10.3%, respectively, being significantly reduced (p<0.01) in the acute phase as compared with 80.7 ± 13.9% in the healthy controls. No significant differences were noted at the onset or in the convalescent phase.

Discussion

CR1 is present on erythrocytes, neutrophils, monocyte/macrophages, B-lymphocytes, some T-lymphocytes, and glomerular epithelial cells). The mean number of E-CR1s is 950/cell, far fewer than neutrophil or lymphocyte CR1s. However, as erythrocytes are more numerous, E-CR1s account for more than 95% of all CR1s in the blood flow, indicating the importance of E-CR1 in the transport and elimination of ICs). We therefore studied time-related changes in the E-CR1 level in patients with acute viral hepatitis, in which ICs are known to appear during the course of the disease.

In healthy controls, the E-CR1 level varied widely, from 56.3 to 100%. Holme et al. determined the number of E-CR1s per erythrocyte in 56 normal individuals by RIA using anti-CR1 antibody and reported 335-8809 sites/cell, showing a wide variation as in our present study. Wilson et al. obtained CR1 genes from normal individuals showing high, middle, and low E-CR1 levels and found that DNA fractions 7.4 Kb

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and 6.9 Kb, obtained by treating the genes with the restriction enzyme Hind III, corresponded to genotypes related to high and low CR1 densities. This suggests that the presence of E-CR1 is genetically regulated.

The E-CR1 level in acute viral hepatitis patients was minimum in the acute phase 2-4 weeks after the onset in AHA and AHB patients but tended to return to the original level in the convalescent period. On the other hand, in 4 of the 7 AHNANB patients, it showed changes similar to those in AHA or AHB patients but remained below 70% in the remaining 3 patients. Of the former 4 AHNANB patients, the E-CR1 level did not decrease even in the acute phase and increased thereafter in one, but it decreased in the acute phase and increased in the convalescent phase in the other 3 as in AHA and AHB patients. In the latter 3 patients, on the other hand, it remained below 70% except for a temporary increase to 75.6% in one. Kondo et al. observed changes in the E-CR1 level in one patient with fulminant hepatitis, 2 patients with drug-induced liver injury, and 2 patients with liver cirrhosis, and stated that the E-CR1 level continued to be low in the cirrhosis patients but decreased in the early stage of the disease and increased in the convalescent phase in those with fulminant hepatitis and drug-induced liver injury. These findings are consistent with our observations if the AHNANB patients who showed consistently low E-CR1 levels are excluded.

In this study, the E-CR1 levels in 7 acute viral hepatitis patients at the onset of the disease, in the acute phase, and in the convalescent phase were compared with those of 8 healthy controls. It was significantly lower in the patients than in the healthy controls in the acute phase.

There have been a number of reports on changes in the E-CR1 level in the clinical course of systemic lupus erythematosus (SLE) and it has been shown to decrease in the active phase of the disease. Wilson et al. reported that the E-CR1 level is genetically determined, but other investigators consider it to be determined by acquired factors on the basis that it is increased in the convalescent. Inada et al. reported that the E-CR1 level and the ICs level are inversely related. Walport et al. noted a rapid decrease in E-CR1 after transfusion of erythrocytes with high CR1 levels in patients with SLE complicated by hemolytic anemia, and suggested that the decrease in E-CR1 in this disease is due to blocking of E-CR1 by autoantibody to CR1 or ICs. In addition, Ross et al. found that C3dg, a degradation product of C3b, was increased on the erythrocyte membrane when E-CR1s were reduced. Since E-CR1 degrades C3b bound by ICs into C3c and C3dg in the presence of an inactivating factor, they considered that increase in C3dg was due to its deposition secondary to the binding of E-CR1 by ICs. These observations suggest that the decrease in E-CR1 in SLE is a result of its blocking by ICs.

Concerning the time of the appearance of ICs in acute viral hepatitis, Aritaka studied its component immunoglobulin classes by Raji cell immunoassay, and noted that IgA and IgM class ICs appeared in the early stage of disease and disappeared within 6 weeks in most cases, but that IgG class ICs reached a peak level 3-6 weeks after the onset in AHA and in the same or slightly later period in AHB, and that no clear peak was noted in AHNANB. This coincidence of the decrease in E-CR1s and the increase in ICs suggests a relationship between E-CR1s and ICs. In addition, Thomas et al. measured ICs by the anti-complementary assay and the C1q binding assay in patients with acute and chronic liver diseases of varying etiologies. They noted the association of increased C1q binding with active inflammation in liver diseases and suggested that increased ICs may indicate that cells of the reticuloendothelial system are disturbed during episodes of acute hepatic necrosis.

From these findings, the significant decrease in the E-CR1 level in the acute phase of acute viral hepatitis is thought to be a phenomenon caused by blocking of E-CR1 by their binding with ICs, which are increased in the blood flow. Therefore, E-CR1 may be involved in the clearance of ICs not only in SLE but also in acute viral hepatitis.

Simultaneous measurement of the E-CR1 and blood IC levels and analysis of components of ICs that bind to E-CR1 are considered to be necessary in the future.
References


急性ウイルス性肝炎患者の赤血球 C3b receptor（E-CR1）についての検討

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要 旨
急性ウイルス性肝炎例（16名）の赤血球 C3b receptor（E-CR1）を ELISA 法を用いて測定した。A 型、B 型、非 A 非 B 型肝炎 13例のE-CR1 値は健常成人に比べ、いずれも急性期では有意に減少。回復期に前値に復した。非 A 非 B 型肝炎の 3 例は E-CR1 値が健常人の平均値以下を保持した。以上より急性ウイルス性肝炎においても E-CR1 が変動し非 A 非 B 型肝炎の中に E-CR1 が持続低値を示すものがあると考えられた。