An Immunoradiometric Assay for Erythrocyte Complement (C3b) Receptor Activity Applied to a Pediatric Population with Connective Tissue Disease

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The number of receptors for complement component C3b per erythrocyte reportedly is decreased in over half of adults with systemic lupus erythematosus. We have devised an immunoradiometric assay for C3b receptor (CR1) on erythrocytes, with which one can assess CR1 saturation due to in vivo binding of immune complexes or activated complement fragments (C3b). Using this assay, we examined binding by CR1 in normal adults and newborns, in lupus and juvenile rheumatoid arthritis patients, and in a population of patients with various general medical problems, including other connective tissue diseases. Binding by CR1 was decreased in eight of 15 SLE patients, four of 25 juvenile rheumatoid arthritis patients, and one of 14 patients with other diseases. We found no significant correlation between CR1 binding and either C1q binding, antinuclear antibody titer, results for complement C3 and C4, or the presence of renal disease. Using this assay, we were also able to show that the observed reduction in CR1 binding was not ascribable to prior saturation of CR1 or to blocking antibody against CR1. The assay is precise and easy enough for routine application.

Additional Keyphrases: pediatric chemistry · immunochemistry · lupus erythematosus · juvenile rheumatoid arthritis

The interrelatedness of compromised function of the reticuloendothelial system, immunoglobulin Fc receptor-mediated splenic clearance, and the connective tissue diseases has been the subject of much interest in recent years. Reduced Fc receptor-mediated clearance in vivo has been observed in active systemic lupus erythematosus (SLE),3 rheumatoid arthritis, and other connective tissue diseases (1–4). It still is unclear whether the observed deficit is a consequence of these connective tissue diseases or a factor preceding them.

Clearance by the reticuloendothelial system that is mediated via complement receptors, in particular the C3b receptor, has received less attention. One study has noted that CR1-mediated clearance is decreased in primary biliary cirrhosis but not in chronic hepatitis and alcoholic cirrhosis (5). Recent in vitro studies of CR1 function suggest that CR1 activity on erythrocytes is defective in a substantial number of adults with SLE (6–8). Two reports have demonstrated that more than half of SLE patients have decreased CR1 activity and that there is a higher incidence of decreased CR1 activity in families of SLE patients than in the general population (6, 8).

However, when anti-CR1 antibody or C3b dimers are used as ligands for measuring CR1 binding, one cannot determine whether to ascribe diminished CR1 binding to prior saturation of CR1 by activated complement component C3 (C3b), to binding of immune complexes, or to blocking antibodies against CR1 (6–8). We report here an immunoradiometric assay for CR1 activity with which one can simultaneously measure saturation or blocking of CR1. We used this assay to study CR1 binding in a pediatric population with connective tissue diseases, including patients with SLE and with juvenile rheumatoid arthritis. No evidence of in vivo saturation was found nor was C1q binding correlated with decreased CR1 activity, confirming previous observations that expression of CR1 is genetically controlled. In addition, our findings suggest that CR1 is also under physiological control with regard to the apparent number of CR1 per erythrocyte.

Materials and Methods

Patients

Blood was drawn after obtaining informed consent in accordance with a protocol approved by our institutions' Institutional Review Committees. The reference population consisted of 17 newborns, from whom cord blood was collected at the time of delivery, and 26 adult laboratory personnel. The patient population included 15 children with SLE, 25 children with juvenile rheumatoid arthritis, and 14 with other conditions. The juvenile rheumatoid arthritis group included seven with systemic disease, nine with polyarticular disease, and nine with pauciarticular disease. All fulfilled the American Rheumatism Association criteria for a diagnosis of juvenile rheumatoid arthritis (9). In the "other diseases" category there were three children with dermatomyositis; one each with psoriatic arthropathy, juvenile ankylosing spondylitis, scleroderma, Crohn's disease, systemic vasculitis, and systemic connective tissue disease; and four other children with general medical problems unrelated to connective tissue disease.

The patients were evaluated both by clinical and laboratory criteria. The degree of disease activity was classified on the basis of the following clinical criteria: arthritis, nephritis, pericarditis, rash, Raynaud's phenomenon, and involvement of the central nervous system. Laboratory studies included complete blood count, platelet count, antinuclear antibody, anti-deoxyribonucleic acid antibody, complement components C3 and C4, and C1q binding.

Procedures

Buffer composition. Buffer A (pH 7.4) contained, per liter, 150 mmol of NaCl, 0.15 mmol of CaCl2, 0.5 mmol of MgCl2, 1 g of gelatin, 3.1 mmol of barbituric acid, and 1.8 mmol of sodium barbital. Buffer B was identical to buffer A except for the addition of 10 mmol of Na2EDTA per liter. Buffer C was prepared by mixing 10 mmol/L solutions of NaHPO4 and NaH2PO4, each containing 0.15 mol of NaCl per liter, to achieve a pH of 7.4.

Collection and preparation of erythrocytes. Blood was drawn into EDTA-containing tubes and the separated erythrocytes were washed twice in buffer B on the same day of collection. The erythrocytes were stored in a volume of

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2 Nonstandard abbreviations: C3b, activated complement component C3; CR1, receptor for C3b; SLE, systemic lupus erythematosus; and AGG, aggregated human immunoglobulin G (IgG).

Received April 22, 1983; accepted June 7, 1983.
Alsever’s solution equal to twice the original blood volume. All studies were carried out within 48 h of collecting the blood. Before testing, the erythrocytes were diluted 10-fold in buffer B and counted in a Coulter S-Plus cell counter. We used about 2 × 10^11 cells/L (or 1 × 10^8 cells/tube) in the assay.

**Preparation of C3b–AGG.** Purified human IgG (15–20 mg/mL), prepared in our laboratory, was aggregated by warming at 63 °C for 30 min, and then diluted in buffer A to a concentration of 150 mg/L. We added 0.5 mL of the diluted IgG to defibrinated plasma diluted 10-fold in buffer A and to defibrinated plasma diluted 10-fold in buffer B. The resulting solution, containing C3b–AGG ligand, was used to measure CR1 activity. Plasma diluted in buffer A contains this ligand; plasma incubated in buffer B contains only the AGG. The ligand was prepared just before each assay and was used the same day.

**Radiolabeling of anti-C3.** The IgG fraction of goat antisera to human C3 (Atlantic Antibodies, Scarborough, ME 04074–0060) was dialyzed overnight against a solution of sodium phosphate (100 mmol/L, pH 7.0) to remove the azide preservative. Its protein content was determined by the method of Lowry et al. (10). We mixed a volume of antiserum corresponding to 1 mg with 1.75 mcI of 125I and 140 μL of lactoperoxidase (EC 1.11.1.7) immobilized on Sepharose (Worthington Biochemicals, Freehold, NJ 07728), according to the method of Janatova et al. (11). Unbound iodide was removed by exhaustive dialysis against buffer C. The specific activity averaged about 9 × 10^8 counts/min per microgram of protein.

**Preparation of C3–Sepharose.** We prepared C3–Sepharose according to the method of Pepys et al. (12), with the following modifications: Human fresh-frozen plasma was substituted for serum and mixed with Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ 08854) in a volume ratio of 5:1, and incubated for 1 h at 37 °C with constant shaking. The gel was filtered through a nylon screen mesh to remove any fibrin formed during incubation, then washed as previously described (12).

**CR1 binding assay.** Erythrocytes from each individual were mixed with both C3b–AGG and AGG in buffer B. Triplicate determinations were done for each ligand solution. Cells, some suspended in the C3b–AGG and others in the AGG solution, were rotated at ambient temperature for 30 min, then washed with three 1-mL portions of buffer C. After the second wash, 0.9 mL of the suspension was transferred to a clean tube for the final wash. The cells were packed by centrifugation and resuspended in 125 μL of 125I-labeled anti-C3 diluted 100-fold in a 10 g/L solution of bovine serum albumin and incubated for 1 h at 37 °C. The cells were centrifuged down and 75 μL of the supernate was added to tubes containing 100 μL of a 300 g/L suspension of C3–Sepharose. This mixture was incubated for 1 h at 25 °C, then washed with three 4-mL portions of buffer containing 5 mL of polyoxyethylene (20) sorbitan monolaurate (Tween 20) per liter, and the bound 125I radioactivity was counted in a Searle 1195 automatic gamma counter. Specific binding was inversely related to the difference in the counts between the binding measured for C3b–AGG and AGG. Normal cells from a single donor were included in each assay. Results were expressed as a percentage of the normal donor binding. The day-to-day variation in binding, measured over 20 days for the same donor, was 9.8% (1032 ± 98 anti-C3 molecules per erythrocyte).

We performed this study using three batches of AGG. The relative number of anti-C3 molecules per erythrocyte varied from 1000 to 2000, presumably reflecting differences in the AGG preparations. However, the CR1 binding for normal controls was not affected, because we used a single donor’s erythrocytes to standardize each assay. Even with the variation noted, the number of anti-C3 molecules per erythrocyte is within the range reported by other investigators for the number of CR1 per erythrocyte (6–8).

**C1q binding assay.** C1q binding was measured in serum of patients and normals by the method of Zubler and Lambert (13). Results are expressed as a percentage of the maximum binding. Normal binding in our laboratory is <8.8% (mean ± 2 SD).

**Trypsin treatment of erythrocytes.** Trypsin (EC 3.4.21.4; Worthington Corp., Freehold, NJ) was purified by affinity chromatography on benzamidine–agarose and stored at -70 °C at a concentration of 5 g/L. Erythrocytes were suspended in 2.0 mL of buffer A to a concentration of 2 × 10^11/mL. To each of four tubes, 25 μL of trypsin was added, and the reaction was stopped in each tube after different intervals by adding soybean trypsin inhibitor (5 g/L, in buffer A). To a fifth tube, the soybean trypsin inhibitor was added before trypsin was added. The erythrocytes were then washed and their CR1 activity was measured. The binding observed for cells treated by the procedures described above was compared with the binding measured in the control tube.

**Statistical analysis.** Student’s t-test of means and the Mann–Whitney U test were used to evaluate the data.

**Results**

**Validation of CR1 assay.** To ensure that the assay described is in fact measuring CR1 activity, several experiments were done to demonstrate both specificity and saturation of binding.

**Dilution of the complement source decreased binding (Table 1), and addition of EDTA to the complement source blocked binding. It is well established that binding to CR1 is sensitive to trypsin activity, whereas the Fc receptor found on leukocytes is not (4). Treatment of human erythrocytes with trypsin decreased binding of the C3b-AGG ligand by 89% (Table 1).

**Saturation of CR1 activity was demonstrated at C3b–AGG concentrations >10 μg/mL in the final reaction mixture (Table 1).**

**C1q binding and other laboratory studies.** C1q binding was assessed in serum drawn simultaneously with sampling for CR1 binding studies. In the neonatal and adult groups, none of 17 and two of 26, respectively, showed C1q binding >8.8%. In the SLE, juvenile rheumatoid arthritis, and other groups, two of 15, three of 25, and one of 14, respectively, were shown to have C1q binding >8.8%. Increased C1q binding and decreased CR1 activity were found in two of eight SLE, none of four juvenile rheumatoid arthritis, and not in one other-disease patient. No positive correlation was demonstrated between increased C1q and decreased CR1 binding (p > 0.05). There was no correlation between binding by CR1 and antinuclear antibody titer, results for complement C3 and C4, or the presence of renal disease.

**CR1 binding in healthy adults, neonates, and children with connective tissue diseases.** The distribution of CR1 binding in neonates and adults overlapped, but the means for each group differed significantly (p < 0.001) (Figure 1). Patients with SLE demonstrated a lower mean binding in comparison to all other groups (p < 0.005). The percentage of patients having CR1 <55% (2 SD below the mean of the combined adult and neonatal populations) was 53.3%, 16.0%, and 7.1% for the SLE, juvenile rheumatoid arthritis, and other-disease groups, respectively.

When the SLE group was subdivided into those with decreased CR1 binding and those with normal CR1 binding,
Table 1. C3b Binding to Human Erythrocytes

A. Variation in C3b-AGG binding to erythrocytes as a function of the dilution of the complement activity of whole serum

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Anti-C3, molecules/cell</th>
</tr>
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<tbody>
<tr>
<td>1:5</td>
<td>1018 ± 25</td>
</tr>
<tr>
<td>1:10</td>
<td>1029 ± 50</td>
</tr>
<tr>
<td>1:20</td>
<td>778 ± 51</td>
</tr>
<tr>
<td>1:40</td>
<td>528 ± 35</td>
</tr>
</tbody>
</table>

B. The C3b-AGG binding to erythrocytes as a function of trypsin treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Anti-C3 molecules/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin + SBTI, 60 min</td>
<td>1810 ± 96</td>
</tr>
<tr>
<td>Trypsin, 10 min</td>
<td>246 ± 96</td>
</tr>
<tr>
<td>Trypsin, 60 min</td>
<td>161 ± 120</td>
</tr>
<tr>
<td>Control*</td>
<td>1560 ± 132</td>
</tr>
</tbody>
</table>

C. Saturation of CR1, binding by C3b-AGG

<table>
<thead>
<tr>
<th>Conc, mg/L</th>
<th>Anti-C3 molecules/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>736 ± 103</td>
</tr>
<tr>
<td>10</td>
<td>1074 ± 54</td>
</tr>
<tr>
<td>50</td>
<td>1116 ± 18</td>
</tr>
<tr>
<td>100</td>
<td>1045 ± 30</td>
</tr>
<tr>
<td>500</td>
<td>1104 ± 49</td>
</tr>
</tbody>
</table>

*Concentration of aggregated IgG in final reaction mixture.

**No preincubation or trypsin treatment.

SBTI, soybean trypsin inhibitor.

Fig. 1. CR 1 binding in adults, neonates, and various connective-tissue diseases

Binding is expressed as percentage of that for a single healthy adult donor. Bars show mean and 2 SD

no significant differences were noted in laboratory data, demographic data, or mean age of the patients. Disease activity as judged by the clinical criteria mentioned earlier was rated as being increased only in the subgroup with decreased CR1 binding. One patient in each SLE subgroup subsequently died of the disease and one in each subgroup had chronic renal failure at the time of the study. Only two of eight patients with decreased CR1 binding had increased C1q binding.

Of those children having juvenile rheumatoid arthritis and decreased CR1 (four of 25), two had systemic and two had polyarticular disease. None of the four patients with decreased CR1 binding had increased C1q binding. Of the remaining juvenile rheumatoid arthritis patients, three of 21 had increased C1q binding. No difference was noted between the mean of results from the juvenile rheumatoid arthritis patients and those patients included in the "other" disease group (p > 0.05).

Several juvenile rheumatoid arthritis and SLE patients (15 of 41) were retested. In all but two cases, CR1 binding was within 12% of the original result. One juvenile rheumatoid arthritis and one SLE patient showed an increase in CR1 binding that placed them within the range of the reference population (>55% binding). Both patients had improved clinically at the time of the second sampling.

In addition, seven of eight SLE and one of four juvenile rheumatoid arthritis patients' sera that demonstrated CR1 binding of <55% were tested for inhibitors of CR1 binding by preincubating normal erythrocytes in 10-fold diluted sera from these patients and then testing for CR1 binding as described. One of seven SLE sera and none of four arthritis sera was found to produce greater than 25% inhibition of binding measured for the same cells preincubated in normal serum. Four additional serum samples from the patient that demonstrated >25% inhibition, collected over a two-year period, did not measurably inhibit CR1 binding activity. (Inadequate serum was available to confirm the initial positive result.) Because prior saturation of CR1 with C3b in vivo was possible cause of decreased CR1 binding in the diseases under study, 125I-labeled anti-C3 binding to erythrocytes in the absence of C3b-AGG was evaluated in all cases. It accounted for <15% of the total binding of 125I-labeled anti-C3b measured in the presence of C3b-AGG in all patients and normal controls.

Discussion

The method described here permits evaluation of the CR1 receptor with regard to prior saturation by activated C3 (i.e., C3b) or by C3b-labeled immune complexes formed in vivo. Immune complexes are relatively common in connective tissue disease. While this procedure does not permit the exact enumeration of CR1 per erythrocyte, the precision of the assay and the ease of reagent preparation make it quite suitable for routine practice. In addition, it can easily be adapted, as shown here, to examination of sera for the presence of anti-CR1 antibody or, further modified, for other anti-receptor antibodies.

Prior studies of CR1 in connective tissue disease have been done primarily on adults with SLE. Miyakawa et al. (8) and Wilson et al. (6) reported decreased CR1 binding on erythrocytes in more than 50% of SLE patients studied and an increased prevalence of decreased CR1 binding in the families of the propositi. Iida et al. (7) also reported decreased CR1 binding on erythrocytes in rheumatoid arthritis, the mean binding for the rheumatoid arthritis group being between that of the normal group and the SLE group.

The findings in each patient category in our study were compared with those for a reference population made up of healthy adults and term newborns with uncomplicated deliveries. The incidence of decreased CR1 binding in the
SLE group was virtually identical to earlier reports on adults (6, 8). Although a greater proportion of the juvenile rheumatoid arthritis patients had decreased CR1 binding as compared with normal, the average binding for the group did not differ significantly from that measured for the combined newborn and adult groups. Average CR1 binding in the "other" disease group was not significantly different from the reference population.

We found nothing indicating that the decreased CR1 binding in the SLE or juvenile rheumatoid arthritis groups is due to either prior saturation of CR1 by C3b or to blocking of CR1 activity by autoantibody binding to CR1.

In agreement with Wilson et al. (6), we found no correlation between decreased CR1 binding and the presence of circulating immune complexes as measured by C1q binding.

When one considers the accumulated data from all studies, the number of CR1 per erythrocyte in any given individual would appear to be under genetic control (6–8). However, several observations suggest that pathophysiological changes may alter the apparent number of receptors per erythrocyte. Two patients in our study and two patients reported by Iida et al. (7) had significantly different CR1 binding on different occasions, which suggests that the average number of CR1 per erythrocyte can change. Two additional pieces of evidence add further support to this hypothesis. First, Wilson et al. (6) have reported that the average CR1 per erythrocyte for macrocytes or younger erythrocytes is higher than the CR1 per erythrocyte in the unfractionated cell population. Second, we have demonstrated a significantly higher CR1 per erythrocyte in newborns as compared with adults. Newborns are known to have a higher percentage of young, macrocytic cells and in addition a shorter erythrocyte life span (15–18). As such, this group presumably represents an example of individuals whose erythrocyte population is shifted toward larger average cell size and hence greater membrane surface area per cell. Because such a shift in the erythrocyte population could influence the apparent CR1 per erythrocyte, then increased erythrocyte turnover—which is not uncommon in connective tissue disease—may produce a shift to higher apparent CR1 per erythrocyte in some individuals. This hypothesis does not exclude genetic control of CR1 expression, but suggests that the average number of CR1 per erythrocyte may vary from individual to individual and within the same individual on the basis of genetic and physiological factors, respectively.

In summary, using a macromolecular ligand for CR1 measurement, we have observed a frequency of reduced CR1 per erythrocyte in children with SLE similar to that reported for adults. A comparatively lower frequency of reduced CR1 per erythrocyte was seen in juvenile rheumatoid arthritis and other connective tissue diseases. Neonates were found to have a significantly higher number of CR1 per erythrocyte than adults, presumably because of their higher proportion of macrocytic erythrocytes. This observation and the finding of higher CR1 per erythrocyte in macrocytes as compared with unfractionated erythrocytes suggests that both erythrocyte turnover and genetic factors regulate the apparent CR1 per erythrocyte. Therefore indicators of cell turnover such as reticuloysis and erythrocyte mean corpuscular volume should be examined concurrently in CR1 studies on erythrocytes.

References