Correlations Between Quantitative Assay of Red Cell-bound C3, Serologic Reactions, and Hemolytic Anemia

By Jörg Th. Fischer, Lawrence D. Petz, George Garraty, and Neil R. Cooper

A new immunochemical method was used to quantitate the number of C3 molecules bound to human red cells in vitro or in vivo and to assess the clinical significance of cell-bound C3 in immune hemolysis. In addition, results utilizing the immunochemical method were compared with those obtained using commonly performed semiquantitative serologic techniques such as the antiglobulin test and antiglobulin titration score. The antiglobulin test using anti-C3 antiglobulin serum became weakly positive with 60-115 molecules C3 per red cell, and was strongly positive with 1000 molecules C3 per red cell. Antiglobulin titration scores correlate well with immunochemical assessment of the number of C3 molecules per red cell. Therefore, a simple extension of the routine antiglobulin test affords clinically useful data concerning the relative degree of sensitization of red cells by C3. Two of fourteen patients with fewer than 1100 molecules C3 per red cell had hemolytic anemia, whereas 8 of 11 patients with greater than 1100 molecules C3 per red cell had overt hemolysis. The presence or absence of hemolysis was not explained by variations in the amount of IgG on these patients’ RBC. It thus appears that the amount of C3 per red cell is an important determinant of hemolysis in human immune hemolytic anemias.

COMPLEMENT COMPONENTS bound to erythrocytes in vivo have been demonstrated in certain patients with immune hemolytic anemias. Using anticomplement antiglobulin (Coombs') reagents, complement components are found on the red cells of all patients with cold agglutinin disease (which comprises about 22% of autoimmune hemolytic anemias), approximately 44% of patients with warm antibody autoimmune hemolytic anemia, and in some patients with drug-immune hemolytic anemias, hemolytic transfusion reactions, and hemolytic disease of the newborn. The demonstration of red cell-bound complement components by the antiglobulin test is important in the detection and precise diagnosis of human immune hemolytic anemias. Such patients also may have low levels of serum complement and an increased fractional catabolic rate of C3, further implicating complement as a mediator of immune red cell destruction in vivo.

The present study applied a new immunochemical method for the quantitation of the number of molecules of red cell-bound C3. The method was stan-

Submitted October 16, 1973; accepted March 19, 1974.
Supported in part by NIH Grant No. NS-09568 and A107007, by the Research Evaluation and Allocation Committee (Gilbert Fund) of the University of California, San Francisco, Calif., and by Eli Lilly & Co. Dr. Fischer was a recipient of a research fellowship of the Heinrich-Hertz Foundation.


Address for reprint requests: Lawrence D. Petz, M.D., Harkness Community Hospital, and Medical Center, 1400 Fell Street, San Francisco, Calif. 94117.

© 1974 by Grune & Stratton, Inc.
dardized by using purified radiolabeled C3 that was quantitated chemically and fixed to red cells or assayed in solution. The amount of C3 on human red cells sensitized in vitro or in vivo was measured with the quantitative assay, and this value was compared with the results obtained using the antiglobulin test and antiglobulin titration score using monospecific anti-C3 antiglobulin serum. Further, the clinical significance of C3 sensitization of red cells in humans was assessed in 25 patients having a positive anti-C3 antiglobulin test. Such findings are discussed in relation to the known pathogenetic mechanisms of complement-mediated immune cytolysis.

MATERIALS AND METHODS

Reagents and Buffers

Freshly prepared Veronal buffer (GVB), pH 7.5, containing 0.1% gelatin, 0.005 M Mg²⁺, and 0.00015 M Ca²⁺ was used. Where ethylenediaminetetraacetic acid (EDTA) was required to block the action of complement, a concentration of 0.01 M was employed. GVB with added EDTA is referred to as GVB-EDTA. 2-mercaptoethanol (2-ME) was obtained from Sigma Chemical Co., St. Louis, Mo.

Complement and Erythrocytes

Normal human serum or fresh guinea pig serum absorbed with human and sheep cells were stored in small aliquots at -50°C and were used as a source of complement. Erythrocytes of various antigenic composition were obtained from normal laboratory personnel. Sheep erythrocytes were obtained from a single sheep from Colorado Serum Company Laboratories, Denver, Colo.

Antiglobulin Reagents

Antiwhole human serum, anti-IgG, and anticomplement component reagents (anti-C3, -C4, -C5, -C6, and -C8*) were prepared in rabbits by multiple subcutaneous injections of the whole serum or isolated proteins together with complete Freund’s adjuvant. All the antisera were thoroughly absorbed with well-washed human group A, B, and O red blood cells and with sheep cells until they no longer reacted with these cells under the conditions of the antiglobulin test. All antiglobulin sera were tested by immunoelectrophoresis with human serum and shown to give only one precipitin line. A single anti-C3 antiserum was used throughout the study. It caused a precipitin line in the anodal region when reacted by immunoelectrophoresis with fresh human serum. When the human serum was reacted with immune complexes or was stored at 37°C for 1 wk, immunoelectrophoresis revealed an arc in a more anodal region and, in addition, an arc characteristic of α₂D.

The anti-C3 antiserum gave negative results when tested for agglutination of normal group A, B, and O red cells with erythrocytes strongly coated with IgG anti-Rh₀(D), and with cells coated with IgM Lewis blood group antibody (anti-Le*). The serum strongly agglutinated red cells sensitized with C3b or C3d (α₂D).

C3

C3 was isolated as has been described and labeled with ¹²⁵I. The concentration of isolated C3 before and after radiolabeling was determined by the Lowry method. Absolute values were obtained by reference to a previously calibrated C3 Lowry curve based on Kjeldahl measurements.

*The complement terminology used in this article conforms to the recommendations of the World Health Organization.
EC3, EA, EAC14, EAC142, and $^{125}$I-C3

Ten milliliters of E (sheep red cells) at a concentration of $1 \times 10^9$ ml were mixed with 10 ml of a 1:20 dilution of human serum as a source of complement which was fixed to the cells by the "naturally occurring" IgM antisheep cell heterophile antibody. Several sera were tested in order to select one which gave optimal coating with C3 without causing excessive lysis. After 10 min at 30°C the cells were washed once with GVB-EDTA in order to stop further complement activity. Then they were washed twice in GVB and resuspended in 10 ml of that buffer to which 0.5 ml of 1.0 M 2ME was added and incubated at 37°C for 20 min. The cells were then washed three times in GVB and stored at 4°C for no longer than 48 hr prior to use. These cells gave a 4+ DAT when tested with a monospecific anti-C3 antiserum. (Further testing with monospecific antisera against C4, C5, C6, and C8 revealed that C4 was also always present. With greater lengths of time of incubation during the first step, complement components C5, C6, and C8 also could be detected.)

EA (sheep erythrocytes sensitized with antibody), EAC14, and EAC142 cells were prepared as previously described.6,17 Radiolabeled EAC142 cells were made by incubating EAC142 cells at 32°C for 20 min with $^{125}$I-C3.

Human Red Cells Sensitized In Vivo With C3

Underlying diseases of patients who had positive direct antiglobulin tests (DAT) with the monospecific anti-C3 antiserum included cold agglutinin disease, paroxysmal cold hemoglobinuria (PCH), drug-immune hemolytic anemia, idiopathic warm antibody autoimmune hemolytic anemia (AIHA), and secondary warm antibody AIHA associated with lymphatic malignancies and systemic lupus erythematosus (SLE). The patient with PCH was previously reported in detail.18 The cells from patients with cold agglutinin disease were always washed carefully at 37°C to disperse autoagglutination before use in the assay.

Human Red Cells Sensitized In Vitro With C3

Ten volumes of neutral, EDTA-treated,19 nonagglutinating anti-Le$^a$ was diluted 1:10 in saline, added to 1 volume of 5% type O Le(a+b-) cells, and incubated for 1 hr at 37°C. The cells were washed four times, and 2 volumes of fresh normal human serum were added. The mixture was reincubated at 37°C for 15 min, and the cells were again washed four times. The amount of complement on the cells at this final stage could be varied by using dilutions of the anti-Le$^a$ in the first stage of the test.

Human Red Cells Sensitized In Vitro With IgG

Commercial anti-Rh$_o$(D) serum (Ortho Diagnostics, Raritan, N.J.) for slide test was diluted 1:4 in saline and an equal volume of 50% type O Rh$_o$(D)-positive cells was added. After incubation at 37°C for 30 min, the cells were washed four times in saline. These cells gave a 4+ antiglobulin test with a monospecific anti-IgG antiserum and were negative using a monospecific anti-C3 antiserum.

Serum C3 Determination

The C3 concentration in normal serum was determined by the radial immunodiffusion technique of Mancini et al.20 A standard solution of known concentration was prepared with purified C3 protein. Three dilutions of the standard solution were used in duplicate in each assay.

Antiglobulin Test

A 1:4 dilution was found to be optimal for all the antisera.19 Two volumes of suitably diluted antiglobulin serum were added to 1 volume of four-times-washed 2% cells in a 10 x 75-mm tube. This tube was centrifuged for 20 sec at 1000 g in a Clay-Adams-Serofuge (Clay Adams, Inc., Parsippany, N.J.). The sedimented cells were gently dislodged from the bottom of the tube, and the presence of agglutination was determined macroscopically over a strong light and graded from $\frac{1}{2}+$ to 4+. The antiglobulin test titration was performed by reacting doubling dilutions...
(1:4 to 1:512) of the anti-C3 antiglobulin serum with C3-sensitized RBC. The titration score was obtained by assigning a value of 8 for 4+ agglutination, 7 for 3½+, 6 for 3+, etc., and determining the sum of the values obtained at each dilution.

C3 Antibody Inhibition Assay

The method used is that of Borsos and Leonard9 and is based on the inhibition of anti-C3 antibody by C3. The C3 to be measured (either cell bound or fluid phase) is added to a standard amount of anti-C3 antibody. Residual antibody activity is then measured by adding the absorbed antiserum to sheep EC3 indicator cells. The EC3-anti-C3 complex fixes complement, and the C3 antibody can be assessed quantitatively by the amount of lysis of the EC3 cells which occurs after addition of guinea pig complement. By comparing the observed lysis with a standard curve obtained with a known concentration of C3 antigen, the amount of test antigen can be measured. The sensitivity of the assay approaches that of radioimmunoassay.9

The lysis of EC3 by guinea pig complement is a function of the concentration of the anti-C3 antisum. A dilution of anti-C3 antiserum was selected that caused approximately 50%, 60%, lysis as follows: 0.2 ml of EC3 (1.5 x 109 ml) was mixed with 0.2 ml of doubling dilutions of anti-C3 antiserum of 1:16 to 1:4000. After 10 min at 30°C, 0.2 ml of a 1:40 dilution of absorbed guinea pig complement was added and the mixture incubated at 37°C for 25 min with appropriate controls. Then 2.5 ml of GVB-EDTA were added, the cells centrifuged, and the amount of lysis was determined by measuring the OD of the supernatant at 412 nm.

The C3 antibody inhibition assay was performed by preparing doubling dilutions of red cells sensitized with C3 in vitro or in vivo in 0.2 ml volumes starting at a concentration of 1.6 x 109 ml. These were mixed with 0.2 ml of optimally diluted anti-C3 antiserum or with GVB buffer as a control. An additional control consisted of anti-C3 antiserum and GVB. The mixture was incubated with occasional mixing for 90 min at room temperature. The suspensions were then centrifuged for 2 min at 3000 rpm in a Serofuge and 0.2 ml of the supernatants incubated with 0.2 ml of EC3 indicator cells at a concentration of 1.5 x 109/ml. The mixture was incubated for 10 min at 30°C. The indicator cells were lysed by adding 0.2 ml of fresh absorbed guinea pig serum at a dilution of 1:40 as a source of complement and incubated for 25 min at 37°C. Controls consisted of EC3 with GVB in place of the supernatant and the guinea pig serum, respectively. 2.5 ml of GVB-EDTA were added to stop the action of complement; the mixture was centrifuged, the supernatants read for lysis at 412 nm, and percentage lysis was calculated using cells lysed in H2O as 100%, lysis.

RESULTS

Standardization of Method

The C3 antibody inhibition assay was standardized three ways, two of which used C3 in solution, and the third used cell bound C3.

C3 of known concentration as determined by radial immunodiffusion in a normal serum was used in preliminary standardization experiments and subsequently as a standard serum in each assay. Figure 1 illustrates the results of a representative C3-antibody inhibition test. The C3 standard serum was run in parallel with the C3-sensitized red cells, and points of equal percentage lysis were compared. Knowing the amount of C3 in the standard serum and the number of C3-sensitized red cells that were used in the inhibition assay, the amount of C3 per red cell may be calculated. For calculations, 185,000 was taken as the molecular weight of C3. The dose-response curve for inhibition by serum C3 was, in general, similar to that obtained for cell-bound C3, and the number of C3 molecules on the test cells was calculated using that dilution of test cells that caused about 50%, inhibition of lysis, since this part of the curve is more reproducible than the extremes of the curve. In the case of weakly sensitized cells, where the maximum amount of inhibition was less than 50%, the
maximum inhibition obtained was used for calculations, providing it was greater than 10%. Less than 60 molecules of C3 per red cell could not be quantitated by this method.

An additional means of standardization of the C3-antibody inhibition test using C3 in solution was performed by measuring the concentration of C3 in the purified $^{125}\text{I}-\text{C3}$ by the Lowry method and comparing this result with that obtained using the C3-antibody inhibition assay. The results of the two assays differed by 9%.

Further, standardization was performed by using $^{125}\text{I}-\text{C3}$ bound to EAC142 cells. Radiolabeled EAC1423 cells were made with approximately 5000-10,000 molecules of C3 per red cell. A 10-20 fold excess of $^{125}\text{I}-\text{C3}$ was reacted with EAC142 cells, since the efficiency of the system is known to be in the range of 5%-10%. Indeed, after correcting for nonspecific absorption (see below), 4.95% of $^{125}\text{I}-\text{C3}$ was found fixed to the red cells.

The cells were then washed three times in GVB and resuspended to a concentration of $1 \times 10^9$ RBC/ml. Doubling dilutions of this suspension were made in 0.2-ml volumes in GVB and counted in a Packard scintillation counter. EA cells were similarly incubated with radiolabeled C3 and assayed in parallel to determine the amount of nonspecific absorption. After measuring the radioactivity, cell-bound C3 was assayed using the C3-antibody inhibition test.

The nonspecific absorption of radioactivity, calculated as the amount of ab-

Fig. 1. A representative C3-antibody inhibition test. Four different kinds of red cells were used for measurement of inhibition of anti-C3 (the unit point representing $1.87 \times 10^7$ red cells). The open triangles represent data obtained with nonsensitized red cells and cells sensitized with IgG (no inhibition in either case). The open and closed circles represent cells from two patients with autoimmune hemolytic anemia sensitized with C3 in vivo. The closed triangles indicate data obtained with cells sensitized in vitro with Le$^a$ antibody and normal human serum as a source of complement. The squares illustrate data obtained when the standard serum served as the source of C3 in the absorption step, the 1-U point representing 14.5 ng C3 (0.2 ml of a 1:12800 dilution of a serum having a C3 concentration of 92.5 mg/100 ml). The calculated number of molecules of C3 per red cell is indicated.
sorption by EA, was 4.4%. After adjustments for background and nonspecific absorption, 7185 molecules of $^{125}$I-C3 per red cell were calculated to be bound to the EAC142 cells. By the inhibition of lysis assay the calculated figure for bound C3 was 6550 molecules C3 per red cell (91%). C3 was not detected on the EA cells by the inhibition of lysis assay in spite of 4.4% absorption of radioactivity.

Red Cells Sensitized With C3 In Vitro

Table 1 summarizes the results of 33 assays of cell-bound C3 done on red cells sensitized in vitro with Le$a$ antibody and varying amounts of complement. The number of C3 molecules per red cell was compared with the results of the antiglobulin test using a single dilution of monospecific anti-C3 antiglobulin serum and with the antiglobulin titration score. The weakest detectable aggluti-

<table>
<thead>
<tr>
<th>Specimen Number</th>
<th>Number of Molecules of C3/RBC</th>
<th>Direct Antiglobulin Test (Anti-C3)</th>
<th>Anti-C3 Antiglobulin Titration Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>115</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>165</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>215</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>340</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>400</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>430</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>435</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>465</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>480</td>
<td>1½</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>480</td>
<td>1½</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>610</td>
<td>1½</td>
<td>7</td>
</tr>
<tr>
<td>12</td>
<td>675</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>13</td>
<td>670</td>
<td>1½</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td>720</td>
<td>1½</td>
<td>7</td>
</tr>
<tr>
<td>15</td>
<td>780</td>
<td>1½</td>
<td>9</td>
</tr>
<tr>
<td>16</td>
<td>790</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>965</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>18</td>
<td>1,145</td>
<td>1½</td>
<td>9</td>
</tr>
<tr>
<td>19</td>
<td>1,190</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>20</td>
<td>1,400</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>21</td>
<td>1,645</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>22</td>
<td>2,670</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>23</td>
<td>2,850</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>24</td>
<td>3,125</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>25</td>
<td>3,300</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>26</td>
<td>4,170</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>27</td>
<td>5,400</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>28</td>
<td>6,450</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>29</td>
<td>10,400</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>30</td>
<td>10,700</td>
<td>3</td>
<td>29</td>
</tr>
<tr>
<td>31</td>
<td>13,650</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>32</td>
<td>16,650</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>33</td>
<td>23,850</td>
<td>4</td>
<td>35</td>
</tr>
</tbody>
</table>
nation in the antiglobulin test was due to 115 molecules of cell-bound C3. There was a progressive increase in the strength of the antiglobulin test and a corresponding increase in the antiglobulin titration score with increasing numbers of molecules of C3 per red cell.

Figure 2 indicates the relationship between the number of C3 molecule per red cell and the antiglobulin test using a single dilution of anti-C3 antiserum. Noteworthy is the fact that a 3+ or 4+ antiglobulin test may result from a wide range of C3 molecules per red cell.

A significant correlation exists between the antiglobulin test titration scores and the quantitative assay of C3 sensitization. After log transformation of the number of C3 molecules per red cell, a linear regression line and the 95% con-
Fidence limits were computed and are illustrated in Fig. 3. The relative increase of red cell-bound C3 per point of antiglobulin test titration score is 16.3%.

**Red Cells Sensitized With C3 In Vivo**

Figures 4 and 5 illustrate the relationship between the number of C3 molecules per red cell and the antiglobulin test or antiglobulin test titration score, respectively. The weakest detectable agglutination in the antiglobulin test was due to 60 molecules of cell-bound C3. As with in vitro sensitized red cells, a strongly positive antiglobulin test may result from a wide range of C3 molecules per red cell.

A significant difference exists between the in vitro and in vivo sensitized red cells that were moderately or strongly sensitized with complement in that for a given antiglobulin test score there were more than four times as many molecules of C3 on in vitro sensitized cells. This difference is expressed in the less steep regression line obtained when plotting the logarithms of the number of C3 molecules per red cell.
molecules per red cell against the antiglobulin test titration score (Fig. 5). For these in vivo sensitized red cells, the relative increase of red cell-bound C3 molecules per point of the antiglobulin test titration score is 7.5%. The differences between in vitro and in vivo sensitized red cells may relate to differences in the nature of the fragments of C3 coating the cells as described below.

Table 2 indicates the correlation of the clinical data of the 25 patients tested in this series with the quantitation of red cell-bound C3. Only two of 14 patients with less than 1100 molecules of C3 per red cell had overt hemolytic anemia as judged by significant anemia in the presence of a persistent reticulocytosis in a patient with no evidence of blood loss. In contrast, eight of 11 patients with greater than 1100 molecules of C3 per red cell did have definite evidence of hemolytic anemia.

DISCUSSION

The anti-C3 inhibition assay for quantitation of red cell-bound C3 affords an opportunity to compare such data with results of existing serologic techniques. Figures 2 and 4 illustrate that a wide range of cell-bound C3 molecules may result in a 2+-4+ direct antiglobulin test, emphasizing that the performance of the antiglobulin test with a single dilution of antiserum is a very crude means of assessing the strength of red cell sensitization. In contrast are the data illustrated in Figs. 3 and 5 which indicate that antiglobulin test scores as calculated in this study correlate quite well with the immunochemical assessment of the number of C3 molecules per red cell. Thus, a simple extension of the routine antiglobulin test affords clinically useful data concerning the relative degree of sensitization of red cells by C3.

Such data is of clinical value since the distinction between the various causes of immune hemolytic anemias is made on the basis of a serologic evaluation which has as one important facet the identification of whether the patient’s erythrocytes are sensitized by immune globulins, complement components, or both. Not only the nature of the protein causing the red cell sensitization but also the strength of this sensitization are significant. For example, as assessed using monospecific antisera against IgG and C3, the red cells of patients with cold agglutinin disease are invariably sensitized with C314 and just as invariably will give a negative direct antiglobulin test when reacted with an anti-IgG serum. In contrast, red cells from patients with Aldomet-21 or penicillin-induced hemolytic anemia22 will invariably demonstrate strong reactivity with anti-IgG and, with rare exceptions, negative reactions are obtained with anti-C3. Immune hemolytic anemia associated with systemic lupus erythematosus is regularly associated with complement sensitization of red cells, usually in association with IgG as well.2 Finally, a small percentage of hospitalized patients similar to many of the patients 1–14 in the present study will have a positive direct antiglobulin test if potent antiglobulin reagents are used. Indeed, in the report of Dacie and Worlledge,2 8% of hospitalized patients had a positive direct antiglobulin test. Such patients characteristically do not have hemolytic anemia, have no abnormal red cell antibodies, and the pathogenesis of the posi-
Table 2. Results of Assays of Red Cell-bound C3 in 25 Patients and Correlation With Clinical and Serologic Data

<table>
<thead>
<tr>
<th>Disease</th>
<th>Anti-C3 Antiglobulin Test (1+ to 4+)</th>
<th>No. C3 Molecules per Red Cell</th>
<th>Anti-C3 Antiglobulin Titration Score</th>
<th>Hemolytic Anemia</th>
<th>Reticulocytes (%)</th>
<th>Anti-IgG Antiglobulin Test (1+ to 4+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Systemic lupus erythematosus</td>
<td>1</td>
<td>60</td>
<td>1</td>
<td>No</td>
<td>41.7</td>
<td>14.8</td>
</tr>
<tr>
<td>2 Iron deficiency anemia</td>
<td>1</td>
<td>90</td>
<td>1</td>
<td>No</td>
<td>21.1</td>
<td>7.0</td>
</tr>
<tr>
<td>3 Normal</td>
<td>1</td>
<td>180</td>
<td>2</td>
<td>No</td>
<td>39.8</td>
<td>14.0</td>
</tr>
<tr>
<td>4 Systemic lupus</td>
<td>1</td>
<td>235</td>
<td>8</td>
<td>Yes</td>
<td>30.2</td>
<td>10.4</td>
</tr>
<tr>
<td>5 Hypertension</td>
<td>2</td>
<td>410</td>
<td>7</td>
<td>No</td>
<td>44.4</td>
<td>15.8</td>
</tr>
<tr>
<td>6 Drug-immune hemolysis (aldomet)</td>
<td>1</td>
<td>465</td>
<td>2</td>
<td>Yes</td>
<td>37.5</td>
<td>12.6</td>
</tr>
<tr>
<td>7 Normal</td>
<td>1</td>
<td>625</td>
<td>2</td>
<td>No</td>
<td>42.0</td>
<td>-</td>
</tr>
<tr>
<td>8 Systemic lupus erythematosus</td>
<td>1</td>
<td>630</td>
<td>3</td>
<td>No</td>
<td>41.5</td>
<td>13.4</td>
</tr>
<tr>
<td>9 Vasculitis</td>
<td>2</td>
<td>700</td>
<td>16</td>
<td>No</td>
<td>40.0</td>
<td>14.1</td>
</tr>
<tr>
<td>10 Systemic lupus erythematosus</td>
<td>2</td>
<td>720</td>
<td>18</td>
<td>No</td>
<td>44.0</td>
<td>14.7</td>
</tr>
<tr>
<td>11 Systemic lupus erythematosus</td>
<td>2</td>
<td>735</td>
<td>15</td>
<td>No</td>
<td>42.2</td>
<td>14.3</td>
</tr>
<tr>
<td>12 Systemic lupus erythematosus</td>
<td>2</td>
<td>1010</td>
<td>8</td>
<td>No</td>
<td>41.7</td>
<td>14.8</td>
</tr>
<tr>
<td>13 Autoimmune hemolytic anemia (warm antibody) (post-splenectomy)</td>
<td>2</td>
<td>1050</td>
<td>6</td>
<td>No</td>
<td>42.0</td>
<td>13.2</td>
</tr>
</tbody>
</table>

The negative antiglobulin test is uncertain. It is typically only weakly positive, and red cell sensitization is usually by complement components only.²

In order to test the clinical significance of cell-bound C3, red cells were obtained from 25 consecutive patients discovered to have a positive direct antiglobulin (Coombs') test that was caused at least in part by red cell sensitization by C3. Only two of 14 patients with less than 1100 molecules of C3 per red cell had hemolytic anemia, whereas eight of 11 patients with at least 1100 molecules of C3 per red cell did have overt hemolysis. In these patients, the presence or absence of hemolysis was not explained by variations in the amount of IgG on these patients' red cells as assessed by the antiglobulin test using monospecific anti-IgG antisera (Table 2). This is well illustrated by patients 17, 19, and 23–25, who had hemolytic anemia without IgG on their red cells detectable by the antiglobulin test, in comparison with patients 5, 20, and 21, who did not have hemolytic anemia but had a strongly positive antiglobulin test using anti-IgG antisera. Although we did not do immunochemical determinations of numbers of IgG molecules on red cells, there is a correlation between the antiglobulin test and such quantitation.²³ Thus, although previous studies have emphasized the role of red cell sensitization by IgG in relationship to red cell survival,²⁴²⁷
it appears that the amount of C3 per red cell is an additional important determinant of hemolysis in human immune hemolytic anemias.

The relationship of complement sensitization of red cells to their in vivo destruction is complex. Complement sensitization of red cells may lead to intravascular hemolysis, extravascular hemolysis, or have no effect on cell survival. The factors which are responsible for these varying effects of complement sensitization include the antibody causing fixation of complement, the biologic activity of the cell-bound complement, the rate of complement fixation, and the number of molecules of complement bound to the cells.

Complement sensitization may result in intravascular lysis. For example, most anti-A and anti-B antibodies are readily hemolytic in the presence of complement in vitro and bring about rapid intravascular hemolysis in vivo. In addition to intravascular lysis, in vivo experiments by Brown et al. demonstrated...
FISCHER ET AL.

strated that red cell destruction may result from the attachment of C3-coated red cells to Kupffer cells with phagocytosis of a proportion of these cells. Other red cells, not phagocytosed, may be returned to the circulation as damaged, spherocytic cells. Such findings confirm similar in vitro phenomena.30-34

In contrast to the above data indicating shortened survival of complement-sensitized red cells, there is evidence that under certain circumstances red cells coated with complement components may survive normally even after temporary sequestration in the liver.35,36 Indeed, red cells of patients with autoimmune hemolytic anemia that are heavily coated with C3 are, in fact, more resistant than normal cells to in vitro or in vivo complement-mediated lysis.37,38 Several factors explain such disparate results regarding the survival of complement-sensitized red cells in vivo. One important consideration is the biologic activity of the complement components on the red cells.

A report in 196640 which assessed biological activity of C3 on human red cells by immune adherence41 demonstrated that cells freshly sensitized in vitro with allo- or autoantibodies and complement are highly reactive by immune adherence, but that erythrocytes from patients with autoimmune hemolytic anemias of warm- or cold-antibody types that are very strongly coated in vivo with C3 as judged by antiglobulin testing are uniformly negative in the immune adherence reaction. Further, incubation of immune adherence-positive cells with serum containing C3 inactivator42,43 (which cleaves cell-bound C3 leaving only C3d [α2D], a hemolytically inert fragment) causes these cells to lose their immune adherence reactivity.44 More recently, Engelfriet has presented serologic data indicating that red cells from patients with autoimmune hemolytic anemia sensitized in vivo with C3 have only C3d (α2D) on their surface,45 and Brown et al.29 and Schreiber and Frank46,47 presented evidence suggesting that the return of complement-sensitized red cells to the circulation from sites of attachment on fixed macrophages is due to the progressive in vivo inactivation of red cell-bound C3.

The rate of complement sensitization is of significance, as indicated by the study of Logue, Rosse, and Gockerman,48 who demonstrated that when a patient with cold agglutinin disease was exposed to cold, there was an acute rise in cell-bound C3 and that this was accompanied by an increased rate of hemolysis. In addition, Frank et al.49 and Schreiber and Frank46,47 have demonstrated that the immunoglobulin class of antibody causing complement sensitization is important, since IgG and IgM antibodies interact with complement in vivo by mechanisms which are qualitatively or quantitatively different and produce different biologic effects.

Finally, as indicated in this study, the number of complement molecules per red cell is an additional important factor regarding cell lysis. Our data are consistent with previous studies of the reaction mechanism of C3 which have specifically indicated that thousands of C3 molecules may be found on red cells lysed in vitro, that the degree of lysis is proportional to the number of C3 molecules bound, and that multiple C3 sites are required for the establishment of a single lytic state.13

It is likely that variability in the above factors, as well as effects of immunoglobulin sensitization of red cells per se,24-27 variability in reticuloendo-
ASSAY OF CELL-BOUND C3

...system functional capacity, explains the lack of precise correlation between the extent of complement sensitization and severity of hemolytic anemia. Nevertheless, the present study indicates that overt hemolytic anemia is much more likely when a patient’s cells are coated with greater than 1100 molecules of C3 per red cell. A large number of molecules of C3 on a red cell surface seemingly reflects a rate of complement sensitization adequately rapid to result in the presence on some red cells of temporarily active complement components in sufficient concentration to lead to shortened cell survival either by direct intravascular hemolysis or via damage by the reticuloendothelial system.

REFERENCES

22. Petz LD, Fudenberg HH: Coombs'-


47. Schreiber AD, Frank MM: Role of antibody and complement in the immune clearance and destruction of erythrocytes. II. Molecular nature of IgG and IgM complement-fixing sites and effects of their interaction with serum. J Clin Invest 51:583, 1972

48. Logue GL, Rosse WF, Gockerman JP: Measurement of the third component of com-
ASSAY OF CELL-BOUND C3

plement bound to red blood cells in patients with the cold agglutinin syndrome. J Clin Invest 52:43, 1973


Correlations Between Quantitative Assay of Red Cell-bound C3, Serologic Reactions, and Hemolytic Anemia

Jörg Th. Fischer, Lawrence D. Petz, George Garratty and Neil R. Cooper

Updated information and services can be found at:
http://www.bloodjournal.org/content/44/3/359.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml