CONCISE REPORT

Increased IgG Molecules Bound to the Surface of Red Blood Cells of Patients With Sickle Cell Anemia

By Lawrence D. Petz, Priscilla Yam, Lee Wilkinson, George Garratty, Bertram Lubin, and William Mentzer

We have used the complement-fixing antibody consumption (CFAC) test to detect small concentrations of IgG on red blood cells from patients with hemolytic anemias that are not thought to be caused by an immune mechanism. Although patients with hereditary spherocytosis, pyruvate kinase deficiency, and mechanical hemolytic anemias generally had normal concentrations of IgG bound to their red cells (~25 molecules IgG per red cell), we found that 39/62 (63%) patients with sickle cell anemia had elevated values. These 39 patients had a mean of 195 and a maximum of 890 molecules of IgG per red cell. None of the patients had been transfused within the previous 90 days, and some had never been transfused. Direct antiglobulin tests were positive in only two instances and autoantibodies were not found in the serum of any patient. However, eluates from the red cells of 6 of 23 patients demonstrated antibody activity against all of a panel of normal red cells by the indirect antiglobulin test. There was no correlation between the number of IgG molecules on patients’ red cells and the severity of their anemia, the incidence of painful sickle cell crises, the reticulocyte count, or with blood transfusion history. We conclude that further study of immunohematologic abnormalities in patients with sickle cell anemia is warranted, especially in view of previous reports in this population of patients with red cell autoantibodies, autoimmune hemolytic anemia, hemolytic transfusion reactions without detectable alloantibodies, and an association of some episodes of pain crises with immunologically mediated red cell destruction.

In an investigation of patients with acquired hemolytic anemia who had a negative direct antiglobulin (Coombs’) test, we used the complement-fixing antibody consumption test to detect small concentrations of IgG on red blood cells. As controls, we used erythrocytes from several groups of subjects, including normal persons and patients with hemolytic anemias that are thought to be caused by nonimmunologic mechanisms. Included among the latter were patients with sickle cell disease, thalassemia major, hereditary spherocytosis, pyruvate kinase deficiency, and mechanical hemolytic anemia caused by the presence of a prosthetic heart valve. Surprisingly, we found that 39 of 62 (63%) patients who were homozygous for hemoglobin S had elevated quantities of IgG bound to their red cell membranes. This article reports these findings and discusses their possible significance in sickle cell anemia.

MATERIALS AND METHODS

Complement-Fixing Antibody Consumption Test

The complement-fixing antibody consumption (CFAC) test described by Gilliland et al. was used to quantitate IgG molecules bound to red cells. A known number of thoroughly washed patients’ red cells were incubated with a predetermined amount of rabbit anti-human IgG. The amount of rabbit anti-IgG not absorbed to the red cells was measured by a quantitative complement fixation test, which, by reference to a standard curve, could be expressed as a quantity of cell-bound human IgG or molecules of IgG per red cell. The latter calculations require the assumption that the binding ratio of rabbit anti-IgG to human cell-bound IgG is the same with antibodies used to develop the standard curve as with other IgG antibodies. The CFAC test is capable of detecting as few as 25 molecules of IgG per red blood cell. The concentration of membrane-bound IgG required for a positive antiglobulin test is variable and is usually in the range of 100-500 molecules per cell.

Serologic Tests

Antiglobulin tests were performed on the red cells of all normal persons and patients using polyspecific antiglobulin serum (Ortho Diagnostics, Inc., Raritan, NJ) as well as anti-IgG and anti-C3 sera prepared and standardized in the authors’ laboratory. All antiglobulin tests were read microscopically following centrifugation. Screening tests for serum autoantibodies were carried out as previously described against both untreated and papainized group 0 cells, with and without a source of fresh complement at 20°C and 37°C. Tests were observed for hemolysis and agglutination at 20°C and 37°C, and an indirect antiglobulin test was performed. Red cell eluates were prepared by either elution and were tested by agglutination and indirect antiglobulin test using normal and enzyme-treated red cells. The specificity of the antibodies was sought by testing several dilutions of the eluate and serum against a red cell panel of varying genotypes, including Rh and cord cells (Spectra Biologies, Inc, and Irwin Memorial Blood Bank).

Subjects

Normal values were derived by performing 88 CFAC tests on 42 hematologically normal subjects who had a negative direct antiglobulin test (DAT).
CFAC tests were performed on patients with nonimmune hemolytic anemias, including 11 patients who had hereditary spherocytosis, one with pyruvate kinase deficiency, and 11 with mechanical hemolytic anemia due to prosthetic heart valves. Also, three patients who had had a splenectomy were tested for comparison with patients with sickle cell anemia, as the latter are known to develop asplenia. None of the splenectomized patients had elevated concentrations of IgG on their red cell surface.

Studies were performed on patients with a variety of hemoglobinopathies who were seen at Children's Hospital in Oakland, Calif., and at Kaiser Permanente Medical Foundation, San Francisco General Hospital, and the University of California Hospital in San Francisco. The diagnosis in each case was established by clinical findings and appropriate hemoglobin analysis. Sickle cell anemia patients were excluded from this study if they had received blood products during the previous 90 days.

A total of 102 CFAC tests were performed on 62 patients with sickle cell anemia. Also, studies were performed on 57 patients with sickle cell trait (hemoglobin A-S). In addition, 14 patients with sickle cell disease (hemoglobin S-C, S-D, and S/β-thalassemia) were studied, as were 6 with homozygous β-thalassemia.

RESULTS

CFAC Tests

The results of the CFAC tests are listed in Table 1. Only one of 88 tests performed on 42 normal subjects revealed an elevated value (48 molecules IgG/RBC). Patients with nonimmunologically mediated hemolytic anemias generally had negative results. Thirteen of 20 tests using red cells from patients with sickle cell disease or β-thalassemia major gave positive results. Although these data are scanty, our results in β-thalassemia are in accord with the report of Galili et al,7 who found IgG on the red cells of 73 of 80 patients with thalassemia.

Most striking were the results obtained in patients with sickle cell anemia. We performed 102 CFAC tests on 62 patients with this diagnosis. A total of 39 of the 62 patients (63%) had increased amounts of IgG on their red cells on at least one occasion. Sixty of the 102 CFAC tests performed were positive (Fig 1), with the median number of IgG molecules per red cell being 290 and the mean being 195. Thirty-one of the positive tests revealed more than 200 molecules of IgG per red cell, with 890 molecules being the maximum value recorded.

In 39 patients, only a single CFAC test was performed; the value was elevated in 22 of these patients and normal in 17.

In order to determine if there were changes in the presence or absence of IgG on patients' red cells, 23 patients had a CFAC test performed on more than one occasion, with the follow-up specimen being obtained from 2 weeks to 4 years after the initial test. Twelve of the 23 patients had 2 CFAC tests, 9 patients had 3 tests, and the other 2 patients had 5 and 6 tests, respectively. Of the 12 patients who had 2 CFAC tests, the results were normal on both occasions in 4 patients and elevated on both occasions in 5 patients. Three patients had normal values initially, but greater than 400 molecules of IgG/RBC when tested 3 months and 3 years later, respectively.

Of the nine patients who had three CFAC tests, five had normal results initially; both follow-up values were elevated in two patients, and one test was elevated in a third. Of the four patients with positive tests on initial examination, six of eight subsequent tests also revealed elevated values.

One of the 23 patients had 5 CFAC tests and another had 6; 9 of these 11 values were elevated.

Seven of the 57 patients (12%) with sickle cell trait yielded abnormal results. The incidence of elevated levels appears to be higher than in normal persons (2.4%), but it is not distinctly different than in patients with hereditary spherocytosis or mechanical hemolytic anemia, wherein 9% of tests were abnormal (Table 1). The significance of these findings is unclear.

Serologic Studies

The direct antiglobulin test was negative in all but two instances. Two patients with sickle cell anemia had weakly positive tests. In one case, the direct antiglobulin test was performed only with polyspecific antiglobulin serum, whereas in the other instance, positive

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of Subjects Tested</th>
<th>Number of Patients With Positive Results*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal persons</td>
<td>42</td>
<td>1</td>
</tr>
<tr>
<td>Hemolytic Anemias</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hereditary spherocytosis</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Pyruvate kinase deficiency</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mechanical hemolytic anemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(prosthetic heart valves)</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Hemoglobin abnormalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sickle cell anemia (S-S)</td>
<td>62</td>
<td>39</td>
</tr>
<tr>
<td>Sickle cell disease (S-C, S-D, S/β-thalassemia)</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>Sickle cell trait (A-S)</td>
<td>57</td>
<td>7</td>
</tr>
<tr>
<td>β-thalassemia major</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

* A positive result indicates >25 molecules of IgG/red cell.
reactions were also obtained with anti-IgG, anti-C3, and anti-C4 antisera. These patients had 263 and 389 molecules of IgG per RBC, respectively, as determined by the CFAC test. It is uncertain why more antiglobulin tests were not positive, as 24 tests revealed more than 300 molecules of IgG per red cell.

Eluates were made from the red cells of 23 patients with sickle cell anemia who had an increased amount of IgG on their red cells. Although only small volumes of blood were generally available for preparing eluates, six eluates demonstrated antibody activity against normal red cells by the indirect antiglobulin test. Two of these eluates were made from the red cells of the patients with a positive direct antiglobulin test. The reactive eluates reacted equally well with all red cells of a red cell panel, with no blood group specificity being evident. More detailed testing was not performed because of the small volume of eluates that were prepared. Autoantibodies were not detected in the serum of any patient.

Correlation Between CFAC Test Results and Clinical Findings in Patients With Sickle Cell Anemia

The patients’ ages varied from 11 months to 30 years. The hemoglobin was 6–11.1 g/dL, and the reticulocytes ranged from 0.8% (during a hypoplastic crisis) to 20%. We have not observed significant correlations between the number of IgG molecules on patients’ red cells and the severity of their anemia, the incidence of painful sickle cell crises, the reticulocyte count, or with blood transfusion history. Red cell survival studies were not performed.

The effect of multiple transfusions on red cell sensitization in patients with sickle cell anemia was considered. Many, but not all, of the sickle cell anemia patients in our population had a history of blood transfusion; however, those who had received blood products in the preceding 3 months were excluded from this study. Therefore, our results cannot be caused by the sensitization of transfused red cells by blood group alloantibodies. Some patients who had never been transfused were among those with increased amounts of IgG on their red cells.

DISCUSSION

Our results indicated that 63% of the patients with sickle cell anemia that we studied had increased concentrations of IgG on their red cell membranes. In two instances, the direct antiglobulin test was positive, but in the other cases, the elevated level of red cell-bound IgG was demonstrated only by the CFAC test, which is more sensitive than the antiglobulin test. A number of investigators have reported that small concentrations of IgG on red cell membranes may not be detected by the antiglobulin test but will be detected by the CFAC test, radiolabelled staphylococcal protein A, radiolabeled anti-IgG, enzyme-linked anti-IgG, or other methods. These low concentrations of red cell-bound IgG may lead to in vivo red cell destruction, as indicated by Mollison and Hughes-Jones who reported that concentrations of an anti-Rho(D) antibody that were too low to be detected by the antiglobulin test were nevertheless capable of shortening red cell survival to approximately 100 hours. Also, numerous cases of acquired hemolytic anemia have been described in which the direct antiglobulin test is negative, although red cell autoantibodies can be demonstrated by other techniques.

The significance of red cell-bound IgG in the sickle cell anemia patients we have studied is at present uncertain. In some cases, the IgG could be eluted from the patients’ red cells and could be shown to react against all normal red cells tested, thus displaying the typical serologic characteristics of a red cell autoantibody. We are unable to state whether or not there is a relationship between the number of molecules of IgG per red cell and the severity of hemolysis, as we did not perform red cell survival studies.

There are several previous reports of the development of red cell autoantibodies in patients with sickle cell anemia. Pirofsky has observed red blood cell autosensitization in patients with sickle cell disease, and Chaplin and Zarkowsky described four patients who developed autoimmune hemolytic anemia superimposed on sickle cell disease and, in addition, reported a fifth patient who had a positive direct antiglobulin test without verified autoimmune hemolysis. Wenz et al described a patient who concomitantly developed red cell autoimmunity and alloimmunization.

Chaplin and Cassell have reported patients with sickle cell anemia who have had apparent hemolytic transfusion reactions although red cell alloantibodies were not demonstrated. We observed a similar patient and suggested that the hemolysis could have been caused by an augmentation of autoantibody production because, in spite of a negative direct antiglobulin test, the patient had 532 molecules of IgG on her red cells at the onset of transfusions, and the red cell eluate demonstrated the presence of an antibody with serologic characteristics indicative of an autoantibody.

Several investigators have commented on the association of sickle cell pain crises with hemolytic reactions, whether caused by red cell alloantibodies or autoantibodies. Thus, one cause of a sickle cell pain crisis may be immunologically mediated red cell destruction.

There are a number of possible mechanisms that may lead to the development of increased concentra-
tions of red cell-bound IgG in patients with sickle cell anemia. An anomalous cell surface structure of sickle cell anemia erythrocytes\(^7\) may be associated with abnormalities of red cell antigens that lead to the development of erythrocyte autosensitization. This could be due to premature expression of the normal “senescence antigen,”\(^18,19\) which we have previously hypothesized may be fundamental to the development of autoimmune hemolytic anemia.2\(^\text{a}\) Similarly, Galili et al\(^7\) have recently described an IgG autoantibody on thalassemic red blood cells. These investigators suggested that the autoantibody may be directed against the age-related antigen present on senescent human red blood cells\(^18,19\) that may be exposed prematurely on thalassemic red cells as a result of abnormal distribution and concentration of sialic acid residues. Finally, patients with splenic atrophy have been reported to have a high frequency of autoantibody.2\(^{11,22}\) This may be relevant to our present studies, as splenic atrophy regularly occurs in sickle cell anemia.\(^23\)

REFERENCES

11. Mollison PL, Hughes-Jones NC: Clearance of Rh-positive red cells by low concentrations of Rh antibody. Immunology 12:63, 1967
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