Red Blood Cell Associated IgG in Normal and Pathologic States

By Irma O. Szymanski, Paul R. Odgren, Normand L. Fortier, and L. Michael Snyder

We studied the anti-IgG-induced agglutination of both normal and abnormal red blood cells (RBC) using a sensitive, automated antiglobulin test. Normal RBC agglutinated strongly with anti-IgG antibody, indicating that IgG was present on the erythrocyte membrane. Young RBC, recovered by centrifugation from a normal RBC population, agglutinated with anti-IgG less than the old cells, suggesting that immunoglobulin G accumulated gradually on the RBC membrane in vivo. The degree of anti-IgG-induced RBC agglutination correlated negatively with the reticulocyte count and positively with the concentration of plasma IgG. RBC from patients with hypogammaglobulinemia appeared to have a low subnormal quantity of membrane-bound IgG, whereas the reverse was the case in hypergammaglobulinemia. During hemolytic episodes, RBC of patients with hereditary spherocytosis agglutinated poorly with anti-IgG, apparently due to predominance of young RBC. Of patients with nonspherocytic, Coombs-negative, nonimmune hemolytic anemia usually also agglutinated poorly with anti-IgG. However, in some cases of active hemolytic anemia, decreased agglutination with anti-IgG was not observed, suggesting that these young RBC had increased amounts of membrane-bound IgG.

Antibodies directed against red blood cell (RBC) membrane components may cause their premature destruction in vivo. This function of RBC-bound antibodies is particularly evident in autoimmune hemolytic anemia. These autoantibodies are usually of the immunoglobulin G variety, and, with or without association of complement components, may elicit a positive direct antiglobulin test (AGT). This test, when performed by the routine manual method and using anti-IgG antibody, is weakly positive if at least 100–150 antibody molecules are present on the RBC. In some cases of immune hemolytic anemia associated with a negative manual AGT, RBC-bound immunoglobulins have been detected by more sensitive methods. On the other hand, normal individuals may have positive direct AGT, but normal RBC survival.

We have employed a sensitive, automated antiglobulin test to study RBC of patients with hemolytic anemia and negative manual direct AGT. Normal RBC and those of patients with hypo- and hypergammaglobulinemia were also tested as a control. In these studies, normal RBC agglutinated strongly with anti-IgG antibody, indicating that immunoglobulin G was bound to their membrane. In contrast, patients’ RBC usually agglutinated less with anti-IgG than normal RBC did. This article reports findings that may explain these somewhat unusual results.

Materials and Methods

Collection and Storage of Blood Samples

Small aliquots. A 10-ml aliquot of whole blood was collected from each of 104 healthy volunteers and from each of 35 patients. The whole blood was anticoagulated either with 1.3 ml of citrate-phosphate-dextrose solution (CPD, Fenwal Laboratories, Morton Grove, Ill.) or with 10.5 mg K3EDTA (Becton-Dickinson Co., Rutherford, N.J.). The blood samples were stored at 4°C and tested within 3 days after collection. In some cases 10-ml aliquots of whole blood were collected with 143 USP U of sodium heparin (Becton-Dickinson Co., Rutherford, N.J.), and tested immediately. In no case did the type of anticoagulant affect the test results. RBC of some samples were cryopreserved with 45% (w/v) glycerol and stored frozen at −80°C until tested. The frozen aliquots were thawed at 37°C and washed free from glycerol initially with 12% NaCl and then with 1.6% and 0.9% NaCl. Freezing, thawing, and deglycerolization did not alter the results significantly. The agglutination of 11 normal RBC samples with anti-IgG was 62.1% ± 5.9% (mean ± 1 SD) prior to freezing and 60.9% ± 7.7% (mean ± 1 SD) after freezing. This difference was not statistically significant (paired t test, p = NS).

Units of blood. From each of 2 healthy blood donors 450 ml of whole blood was collected into a plastic bag containing 63 ml of citrate-phosphate-dextrose solution (CPD). Most of the visible plasma was removed following centrifugation in an RC-3 refrigerated centrifuge (Ivan Sorvall, Norwalk, Conn.) and the RBC was saved for further testing.

Patients With Hypo- and Hypergammaglobulinemia

RBC from each of eight patients with hypogammaglobulinemia were studied. The etiology of this condition was congenital in three cases (courtesy of Dr. F. S. Rosen, Children’s Hospital, Boston, Mass.). One patient had x-linked recessive “lymphoproliferative” syndrome (courtesy of Dr. D. Purtilo, U/Mass Medical School, Worcester, Mass.), and two other patients were suffering from chronic lymphocytic leukemia. Another two patients had untreated multiple myeloma with Bence-Jones proteinuria (lambda chain) associated with hypergammaglobulinemia. Three patients had elevated IgG levels because they suffered from IgG myeloma. Two patients had elevated IgG levels due to chronic inflammation.

Patients With Hereditary Spherocytosis

RBC from each of ten patients with hereditary spherocytosis were studied. The severity of the disease varied among the patients, but all had presented with splenomegaly; they had spherocytes in
Peripheral blood (low mean corpuscular volume [MCV], increased mean corpuscular hemoglobin concentration [MCHC], and abnormal morphology), and the osmotic fragility of freshly drawn blood was increased. Three patients had been previously splenectomized, after which they were neither anemic nor showed signs of increased RBC destruction. Two of the patients were studied before and after splenectomy. The remaining five patients had splenomegaly, and they showed evidence of rapid red cell destruction (e.g., reticulocytosis and indirect bilirubinemia).

**Patients With Hemolytic Anemia of Various Etiologies**

RBC of each of 12 patients with nonspherocytic hemolytic anemia having negative manual direct AGT were studied. In three patients, the etiology of the condition was not known, and three were suffering from glucose-6-phosphate dehydrogenase (G6PD) deficiency associated with chronic hemolytic anemia. Four patients had hereditary xerocytosis, one had paroxysmal nocturnal hemoglobinuria (PNH), and one had sickle cell disease.

The patient with PNH had increased transfusion requirements. In this patient, the survival of donor RBC was assayed with an automated differential agglutination technique.

**Routine Laboratory Tests**

Coulter counter was used to record hemoglobin concentration and hematocrit. Reticulocytes were stained with new methylene blue, and the percentage was determined following enumeration of 1000 RBC. Serum IgG concentration was measured by radial immunodiffusion method. Osmotic fragility was determined by the Standard Method. The manual direct antiglobulin test was done by routine method using broad spectrum reagent (Ortho Diagnostics, Raritan, N.J.).

**Direct Antiglobulin Test Using the Auto Analyzer**

Agglutination of RBC with anti-IgG was measured in the Auto-Analyzer (Technicon Corporation, Ardsley, N.Y.) as described previously. We used approximately 20% (v/v) suspensions of RBC in 0.5% (w/v) Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) and 0.9% (w/v) sodium chloride. One percent (w/v) PVP K90 (polyvinylpyrrolidone, K90, Technicon Corporation, Ardsley, N.Y.) in 0.9% NaCl was used to enhance the agglutination, and anti-IgG antiserum was diluted with 0.5% bovine serum albumin (Reheis Chemicals, Phoenix, Ariz.) and 0.9% NaCl. Two-hundred-thousand times diluted antiserum agglutinated normal RBC only slightly, although "Coombs"-positive RBC agglutinated strongly with this dilution of antiserum. "Maximal agglutination" was obtained with 5000 times diluted anti-IgG. Further increases in the strength of the antiserum decreased the percentage agglutination slightly (prozone phenomenon), indicating that the agglutination at the maximal point was limited by the reactivity of RBC and not by the amount of antibody.

In this study we characterized RBC populations by determining their maximal agglutination value with anti-IgG. It was considered that the higher the agglutination, the more there was RBC-bound IgG, and vice versa. This is shown in the following experiment. Five 0.5-ml aliquots of Rh(D)-positive RBC obtained from a hypogammaglobulinemic individual were each incubated at 37°C with various quantities of commercial anti-D (Ortho Diagnostics, Raritan, N.J.). The degree of RBC sensitization with anti-D was assumed to be proportional to the quantity of anti-D added to the RBC samples. The sensitized RBC aliquots were washed and tested for agglutination with anti-IgG using both manual and automated tests. All samples had a negative manual direct antiglobulin test. Figure 1 shows that when RBC were incubated with increasing amounts of anti-D (IgG), their maximal agglutination value increased higher in the automated test.

The maximal agglutination value of a particular red blood cell population with anti-IgG was reproducible. Tests with replicate samples showed that the coefficient of variation was, on the average, 0.93% (n = 4). Tests with 4 samples of RBC, using anti-IgG, revealed a mean variance of 1.046 due to replications (n = 10) within samples and a variance of 146.79 between the means of samples (p < 0.001 by analysis of variance).

**Antiserum**

Anti-IgG antiserum (Fc fragment specific) was obtained from Atlantic Antibodies (Westbrook, Me.), lot JGG-PO49-N. This serum did not contain anti-IgA or anti-IgM antibodies. Heteroagglutinins were removed from the antiserum by incubating 1 vol of serum with 1/10 vol of fresh RBC (a mixture of blood types O, A, and B) at 22°C for 0.5 hr while the sample was constantly mixed. The removal of heteroagglutinins was verified both by manual and automated tests. In manual tests, the absorbed, undiluted serum did not agglutinate normal RBC. In the automated test, the removal of heteroagglutinins could be verified only following removal of the anti-IgG antibody. This was accomplished by the method of immunoabsorption.

The immunoabsorbent was prepared by coupling Cohn Fraction II, 3 (containing 15.3 g/dl IgG, 200 mg/dl IgA, and 340 mg/dl IgM obtained from Mr. Larsen at the Massachusetts Department of Public Health, State Laboratory Institute, Jamaica Plains, Mass.) to cyanoagen-bromide-activated Sepharose 4B (Sigma Chemical Co., St. Louis, Mo.). In the automated tests, the unabsorbed serum (but anti-IgG removed) agglutinated normal RBC when diluted 80 times, whereas the undiluted, absorbed serum (anti-IgG removed) failed to agglutinate either normal RBC, or RBC coated with anti-D antibodies.

**Effect of RBC Age**

Twenty-milliliter aliquots of heparinized whole blood collected from each of 6 volunteers were separated into light (young) and...
The percent agglutination of the RBC was determined by determining the RBC indices and reticulocyte counts. Supernatant fluids were tested for their ability to dilute normal serum. Following 30 min of incubation at 37°C, the 0.9% NaCl (standard), and with 1,000,000 and 500,000 times was also combined with times. An equal volume of diluted anti-IgG suspension was incubated with 0.9 ml of anti-IgG, diluted 500,000 counts.

Neutralization of Anti-IgG Antibody

Ten percent suspensions were prepared from: (A) washed, nontreated RBC; (B) from washed, bromelinized RBC; and (C) from washed, bromelinized and plasma-treated RBC. All the RBC counts were adjusted to be equal. One milliliter of each RBC suspension was incubated with 0.9 ml of anti-IgG, diluted 500,000 times. An equal volume of diluted anti-IgG was also combined with 0.9% NaCl (standard), and with 1,000,000 and 500,000 times diluted normal serum. Following 30 min of incubation at 37°C, the supernatant fluids were tested for their ability to agglutinate sensitized RBC in the AutoAnalyzer. The RBC sensitization was accomplished by incubation of a 50% RBC suspension with 1/10 vol of anti-D (Ortho Diagnostics, Raritan, N.J.). The sensitized RBC produced 2+ reaction by the manual AGT. The percent neutralization of anti-IgG antiserum was calculated with the aid of the following formula:

\[
\text{Percent neutralization} = 100 \times \frac{\text{OD test} - \text{OD standard}}{\text{OD Alb} - \text{OD standard}}
\]

where OD is the optical density at 550 nm and Alb is 0.5% albumin substituted for anti-IgG.

<table>
<thead>
<tr>
<th>Table 1. Agglutination of “Young” and “Old” RBC With Anti-IgG</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Top layer</td>
</tr>
<tr>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Bottom layer</td>
</tr>
<tr>
<td>Mean ± SD</td>
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<tr>
<td>Paired t</td>
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</tbody>
</table>

Six (6) RBC populations were separated into “young” and “old” cells by centrifugation. Red blood cell indices and reticulocyte counts for the separated populations are also shown.

RESULTS

Agglutination of Normal RBC With Anti-IgG

Washed RBC suspensions obtained from peripheral blood of normal persons agglutinated strongly with anti-IgG. On the average, the agglutination was 65.7% ± 5.7% (mean ± 1 SD) (n = 104).

When normal RBC were separated on the basis of their density, the agglutination with anti-IgG of the RBC located in top layer was 53.2% ± 5% (mean ± 1 SD) (n = 6), and the agglutination of the RBC located in the bottom layer was 68.5% ± 3.03% (mean ± 1 SD) (n = 6). The difference in agglutination of the two RBC populations was statistically significant (p < 0.002). MCV, MCHC, MCH, and reticulocytes of these separated populations are shown in Table 1, and they reflect appropriate separation of RBC by density.

The second centrifugational separation permitted isolation of an RBC population with an average reticulocyte count of 4.6%. The agglutination of these RBC with anti-IgG was, on the average, 36.0%.

Effect of RBC Age

Figure 2 shows that there was statistically significant negative correlation between the reticulocyte count and the magnitude of anti-IgG-induced agglutination of an RBC population (r = –0.641, n = 45, p < 0.002). RBC from normal subjects, separated by differential centrifugation into reticulocyte-rich and reticulocyte-poor populations, and those from patients who had accelerated RBC destruction but normal concentration of plasma IgG were analyzed in this manner.

Effect of Proteolytic Enzymes

Following treatment of several normal RBC with either bromelin or trypsin, they did not agglutinate with anti-IgG. In one typical experiment, 10% suspensions of well washed, normal RBC neutralized about 25% of the agglutinating activity of anti-IgG (1,000,000 times diluted), whereas bromelinized RBC neutralized only 2% of the agglutinating activity of...
Patients With Hypo- and Hypergammaglobulinemia

In patients with hypogammaglobulinemia, the agglutination of RBC with anti-IgG was subnormal, $31.2\% \pm 10.7\%$ (mean $\pm$ 1 SD). These data are shown in Fig. 3. Following incubation of these RBC in normal plasma, the agglutination increased only slightly, about 3%. None of these patients had signs of increased RBC destruction, but two patients were anemic because of underlying bone marrow malignancy.

On the other hand, RBC of patients with hypergammaglobulinemia agglutinated with anti-IgG more than normal RBC. Figure 4 shows that significant correlation was observed between the concentration of plasma IgG and the magnitude of anti-IgG-induced RBC agglutination ($r = 0.623, n = 26, p < 0.002$). RBC from normal subjects and those from hypo- and hypergammaglobulinemic patients were included in these data. Although the relationship between these two variables was exponential, a linear relationship was observed in the initial part of the curve, coinciding with plasma IgG concentration less than 1000 mg/dl ($r = 0.858, n = 15, p < 0.002$).

Patients With Hereditary Spherocytosis

Figure 3 shows the magnitude of RBC agglutination with anti-IgG antibody in 10 patients with hereditary spherocytosis. RBC of 6 patients who had not had splenectomy agglutinated poorly with anti-IgG, the mean $\pm$ 1 SD being $30.4\% \pm 21.8\%$. RBC of patients who had only mild reticulocytosis agglutinated almost
normally, whereas RBC of those patients who had excessive reticulocytosis agglutinated poorly. Plasma IgG concentration was measured in 4 of these 6 patients, and it was below the normal adult range in one (440 mg/dl). This 6-mo-old patient had only mild hemolysis and a reticulocyte count of 4.2%. Nevertheless, RBC agglutination with anti-IgG was only 15.9%. It appears that both hypogammaglobulinemia and hemolysis contributed to the poor agglutination. RBC of another patient agglutinated only minimally (0.5%) with anti-IgG. This 3.5-yr-old patient had a hematocrit of 25% and a reticulocyte count of 12%. The plasma IgG concentration was low normal (700 mg/dl). In contrast, the agglutination of RBC with anti-IgG in those patients who had had splenectomy was almost normal, 58.2% ± 7.3% (mean ± 1 SD).

**Effect of Splenectomy**

Two siblings of the same family (K.V. and D.V.) having hereditary spherocytosis were tested before and after splenectomy. Figure 5 shows that after splenectomy, normal hematocrit and reticulocyte count were observed within 1 wk, whereas the agglutination of RBC with anti-IgG normalized more slowly.

**Patients With Other Types of Hemolytic Anemia**

The diagnosis and laboratory values of patients suffering from nonspherocytic hemolytic anemia are shown in Table 2. On the average, the agglutination of RBC with anti-IgG was 40.2 ± 17.3% (mean ± SD). These values are compared to the normal values in Fig. 3. As a rule, decreased agglutination of RBC with anti-IgG was observed despite normal or high concentrations of plasma IgG.

Three patients had RBC that agglutinated normally with anti-IgG. The exact etiology of the hemolysis in two cases was not known. The third case had been diagnosed as paroxysmal nocturnal hemoglobinuria. She had increased transfusion requirements, but no irregular antibodies had been demonstrated in her serum by manual methods. The survival of donor RBC, measured by an automated differential agglutination technique, was decreased, the lifespan being 55 days (normal about 100 days).

**DISCUSSION**

We have used a sensitive automated antiglobulin test to study the agglutination of normal and abnormal RBC with anti-IgG antiserum. In these tests, 5000 times diluted antiserum agglutinated RBC maximally,
indicating that IgG was present on any RBC membrane. This conclusion was confirmed by tests that showed the agglutination reactions to be specific for cell-bound IgG, since no agglutination was observed following removal of the anti-IgG component from the antiserum by immunoabsorption. Experiments with RBC sensitized with several subagglutinating quantities of anti-D also showed that the more the RBC had been sensitized, the more they agglutinated with anti-IgG (Fig. 1). Although not generally appreciated, several investigators using very sensitive methods had previously detected IgG on normal RBC.

The capacity of RBC of healthy persons to agglutinate under the conditions of our test was remarkably similar. We observed that the young RBC of normal persons agglutinated significantly less than the old RBC. In addition, we could show that there was a statistically significant negative correlation between percentage reticulocytes in peripheral blood and percentage agglutination of RBC with anti-IgG. The magnitude of anti-IgG-induced agglutination, therefore, reflects mean RBC age. These differences between young and old RBC could indicate differences either in RBC agglutinability or in the density or quantity of RBC-bound IgG. We prefer to think that young RBC have less membrane-bound IgG than old cells. Utilizing electronmicroscopic techniques Kay has observed IgG molecules on the membrane of old, but not of very young, RBC, thus lending support to our interpretation.

Treatment with the proteolytic enzyme bromelin destroyed the ability of RBC to agglutinate with or neutralize anti-IgG but not their ability to reabsorb IgG from autologous plasma following a short incubation period. These data indicated that bromelin destroyed RBC-bound IgG but not the receptor for IgG. In contrast, it appeared that in vivo a period of several days was necessary for young RBC to acquire IgG. It is possible that IgG "receptors" on RBC membrane become gradually exposed in vivo, resulting in IgG binding. The exact function of these cell-bound immunoglobulins remains unclear. It is possible that they have no biologic function. It is known that even large amounts of IgG can be present on the RBC membrane without affecting cell survival. One could also consider the possibility that RBC-bound IgG protects against cell destruction by covering the antigenic sites that become exposed in vivo, thus acting as "enhancing" antibodies. Kay, on the other hand, maintains that RBC-bound IgG is a physiologic autoantibody, which eventually induces the elimination of the senescent erythrocytes.

In addition to mean RBC age, plasma IgG concentration also affected the anti-IgG-induced agglutination of RBC, and a significant positive correlation was observed between these two variables. Therefore, it should be noted that the percentage agglutination of RBC with anti-IgG reflects mean RBC age only in those individuals who have normal concentrations of plasma IgG.

Although the automated antiglobulin test is very sensitive and can detect IgG on normal RBC, the existence of the normal IgG "background" can interfere with the detection of small amounts of cell-bound allo- and autoantibodies. Discrimination between normal and pathologic IgG on RBC is further complicated by the finding that the anti-IgG-induced agglutination depends both on plasma IgG concentration and cell age. Since during hemolytic episodes the mean age of a RBC population is young, one would expect the anti-IgG-induced agglutination to be appropriately decreased in nonimmune hemolytic anemias. In hereditary spherocytosis, a nonimmune disease, this indeed was the case. Effect of splenectomy was dramatic in two patients. Hemograms

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**Table 2. Agglutination of RBC With Anti-IgG and Other Laboratory Data in Patients With Hemolytic Anemia of Various Etiologies**

<table>
<thead>
<tr>
<th>Initials</th>
<th>Etiology</th>
<th>RBC Count (10^6/μl)</th>
<th>MCV (μm3)</th>
<th>Reticulocyte Count (%)</th>
<th>Serum IgG (mg/dl)</th>
<th>Agglutination With Anti-IgG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.W.</td>
<td>Idiopathic</td>
<td>2.41</td>
<td>105</td>
<td>9.1</td>
<td>1,500</td>
<td>65.4</td>
</tr>
<tr>
<td>J.D.</td>
<td>Idiopathic</td>
<td>2.78</td>
<td>106</td>
<td>11.8</td>
<td>ND</td>
<td>58.8</td>
</tr>
<tr>
<td>M.H.</td>
<td>Idiopathic</td>
<td>2.84</td>
<td>106</td>
<td>13.9</td>
<td>970</td>
<td>47.3</td>
</tr>
<tr>
<td>A.S.</td>
<td>PNH*</td>
<td>2.19</td>
<td>102</td>
<td>8.9</td>
<td>790</td>
<td>67.0</td>
</tr>
<tr>
<td>R.R.</td>
<td>Xerocytosis</td>
<td>4.48</td>
<td>97</td>
<td>7.2</td>
<td>1,300</td>
<td>28.4</td>
</tr>
<tr>
<td>J.R.</td>
<td>Xerocytosis</td>
<td>3.63</td>
<td>99</td>
<td>8.5</td>
<td>1,850</td>
<td>48.7</td>
</tr>
<tr>
<td>Ro.T.</td>
<td>Xerocytosis</td>
<td>4.61</td>
<td>93</td>
<td>7.5</td>
<td>1,000</td>
<td>23.2</td>
</tr>
<tr>
<td>R.T.</td>
<td>Xerocytosis</td>
<td>4.5</td>
<td>93</td>
<td>6.5</td>
<td>ND</td>
<td>25.7</td>
</tr>
<tr>
<td>L.P.</td>
<td>G6PD def.</td>
<td>3.2</td>
<td>116</td>
<td>10.8</td>
<td>ND</td>
<td>33.1</td>
</tr>
<tr>
<td>D.P.</td>
<td>G6PD def.</td>
<td>3.31</td>
<td>112</td>
<td>18.1</td>
<td>1,000</td>
<td>13.8</td>
</tr>
<tr>
<td>R.Z.</td>
<td>G6PD def.</td>
<td>3.45</td>
<td>122</td>
<td>18.5</td>
<td>1,480</td>
<td>31</td>
</tr>
<tr>
<td>J.C.</td>
<td>Sickle cell disease</td>
<td>3.29</td>
<td>93</td>
<td>18.0</td>
<td>1,650</td>
<td>39.8</td>
</tr>
</tbody>
</table>

**ND. not done.**

*Patient had increased transfusion requirements.*

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normalized rapidly after splenectomy, while RBC agglutination normalized more slowly.

RBC of patients with nonpherocytic nonimmune hemolytic anemia also agglutinated poorly with anti-IgG. In these cases, the subnormal agglutination did not reflect low plasma IgG (Table 3), but probably indicated that either the quantity of the RBC-bound IgG was low or that the “agglutinability” of the abnormal RBC was impaired. Disturbances of RBC agglutination are difficult to determine, but might be indirectly estimated by quantitating RBC-bound IgG.

RBC of three patients with hemolytic anemia agglutinated “normally” with anti-IgG, although the RBC populations obviously were young. This suggested that higher than normal amounts of cell-bound IgG might be present. Although the etiology of hemolysis was unknown in two of these cases, autoimmunity could have been a contributing factor. In the patient with PNH, the survival of donor RBC was decreased, possibly due to the action of autoantibodies.

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Red blood cell associated IgG in normal and pathologic states

IO Szymanski, PR Odgren, NL Fortier and LM Snyder