Demonstration of a Natural Antigalactosyl IgG Antibody on Thalassemic Red Blood Cells

By Uri Galili, Aviva Korkesh, Itzhak Kahane, and Eliezer A. Rachmilewitz

A modified antiglobulin test, based on the high affinity between the Fc portion of the red blood cell (RBC) bound IgG and the Fc receptor on the myeloid cell K-562, was utilized for demonstration of immunoglobulins (Ig) on thalassemic RBC. Ig was found on the RBC of 73 out of 80 patients with thalassemia. The immunoglobulins on the thalassemic RBC belonged to the IgG subclass and were autoreactive. Elution studies utilizing various carbohydrates, or by thermal stripping, indicated that at least part of the IgG molecules found on the thalassemic RBC were specifically reactive with terminal galactosyl residues on the RBC membrane. IgG antibodies with similar reactivity were also demonstrated in normal human serum. These natural antigalactosyl IgG antibodies from normal sera could bind to IgG-depleted thalassemic RBC. Thalassemic RBC and normal senescent RBC were previously found to contain reduced amounts of membrane sialic acid (SA). It is suggested that the antigalactosyl IgG antibodies interact with newly exposed galactosyl residues underlying the sialic acid units. Such interaction may lead to the shortened lifespan of thalassemic RBC and may result in sequestration of senescent normal RBC by the reticuloendothelial system.

ULTRASTRUCTURAL STUDIES have demonstrated that sialic acid (SA) residues on the surface of thalassemic red blood cells (RBC) are unevenly distributed and are less abundant when compared to those on the surface of normal RBC. In addition, biochemical data indicated that the SA level in the glycophorin portion of thalassemic RBC is approximately 25% less than in normal RBC membranes. Since young thalassemic RBC have comparable SA levels to young normal RBC, the low SA levels found in unfractonated thalassemic RBC has been attributed to an enhanced rate of SA removal by an unknown mechanism. The short lifespan of the thalassemic RBC may be related to increased rate of sequestration due to reduction of surface SA. The loss of membrane SA may result in exposure of hidden antigenic sites capable of binding autologous natural IgG antibodies. Studies on normal human, rabbit, and rat RBC indicated that the removal of SA by neuraminidase resulted in exposure of antigens that bound autologous IgG, with subsequent phagocytosis of the treated RBC in vivo or in vitro. Moreover, aging of normal RBC is also associated with reduction of SA and binding of autologous IgG to the newly exposed antigens. The bound IgG molecules facilitate the sequestration of the senescent RBC by the reticuloendothelial phagocytic cells.

In view of these findings, the short lifespan of the thalassemic RBC may be visualized as an acceleration of the normal RBC aging process. The purpose of the present study was to demonstrate possible in situ binding of IgG molecules to newly exposed antigens on the SA-reduced thalassemic RBC. For detection of IgG bound to thalassemic RBC, we utilized a sensitive qualitative antiglobulin test. The results indicated that RBC from most thalassemic patients bear autologous IgG antibodies. Studies on the specificity of these antibodies showed that they are directed toward terminal galactosyl residues that seem to be exposed following removal of SA in situ.

MATERIALS AND METHODS

Blood Source

Eighty thalassemic patients (45 males and 35 females) were studied. Most of the patients were Kurdish Jews and some were Arabs. Seventy-two patients suffered from β-thalassemia major, and 8 patients from β-thalassemia intermedia. Their age ranged between 5 and 35 yr. Seventy patients were splenectomized. Sixty-six patients are regularly transfused every 4–8 wk, and the remainder have rarely received a blood transfusion or have received no transfusion at all. Detailed clinical and laboratory findings of these patients have been previously reported. All blood samples were taken prior to blood transfusion, when required. RBC from 50 healthy individuals of the same age group served as control. The sera from 20 healthy individuals and 30 cord blood samples were analyzed for antigalactosyl antibodies.

Direct Antiglobulin Test by EA Rosettes (EA-DAT)

A quantity of 0.1 ml of 1% suspension of washed RBC was mixed with 0.1 ml rabbit anti-human serum (broad spectrum containing anti-IgG, IgM, IgA and anticomplement, Ortho Diagnostics, Raritan, NJ) and incubated for 30 min at 24°C. Thereafter, the RBC were washed twice and mixed with 0.1 ml K-562 cell suspension in saline (106 cells/ml). This myeloid cell line, originally described by Lozzio and Lozzio, expresses the Fc receptor for antigen-bound IgG molecules. The mixed suspension of K-562 cells and RBC was spun for 5 min at 200 g and incubated for 30 min at 4°C. The pellet was resuspended, and the percentage of K-562 cells binding IgG-
coated RBC and forming EA rosettes was scored in a hemocytometer. This method was found to be 20–50-fold more sensitive than the regular antiglobulin agglutination assay. Furthermore, the rosette scoring enables a relative quantitation of the Ig level on the tested RBC.

In a series of assays specific rabbit anti-human IgG and rabbit anti-human IgM (Ortho Diagnostics) and specific rabbit anti-kappa and anti-lambda chains (Dako, Denmark) were utilized in parallel to demonstrate its binding to RabRBC followed by specific elution with antigalactosyl activity of the dialyzed IgG was confirmed by demonstrating its binding to RabRBC followed by specific elution with galactose.

Elution of RBC-Bound IgG

The elution of the IgG was performed in two different methods. (A) Specific elution by incubation of the RBC at 37°C for 30 min, in solutions of 0.15 M of various carbohydrates. The RBC-bound Ig with anticalbohydrate activity are eluted by the specific carbohydrate present in excess in the solution. (B) Nonspecific elution by heat. A 50% suspension of thalassemic RBC in saline was incubated for 2 hr at 45°C. Following this treatment, the membrane-bound IgG was eluted, as indicated by the diminished capacity of the RBC to form EA rosettes. The suspensions were spun at 1,000 g for 2 min, and the supernatants were further assayed for anti-rabbit RBC (RabRBC) antibodies with specificity for galactosyl residues.

Treatment of Normal RBC With Vibrio cholera Neuroaminidase (VCN)

A 5% suspension of washed human RBC (HuRBC) in RPMI 1640 medium was incubated with 10³ U/ml of VCN solution (Behringwerke, Mannheim, Germany) for 30 min at 37°C. The RBC were washed and assayed for antibody binding in autologous serum and cord blood serum by the EA-IDAT.

RBC Separation to Different Age Populations

RBC were separated by density centrifugation into different age populations according to the method of Murphy. The 10% top (young) and bottom (old) fractions were assayed for EA-DAT.

Isolation of Antigalactosyl IgG From Normal Sera

Two milliliters of packed RabRBC were incubated for 30 min at 37°C with an equal volume of heat-inactivated normal serum, formerly found to contain antigalactosyl IgG (see Results section). Thereafter, the RabRBC were washed 3 times with saline and resuspended in 0.15 M galactose solution to a final volume of 4 ml. The suspension was incubated for 30 min at 37°C. The supernatant containing eluted IgG bound to galactose was dialyzed twice against 2 liters of saline for the removal of galactose molecules. The antigalactosyl activity of the dialyzed IgG was confirmed by demonstrating its binding to RabRBC followed by specific elution with galactose.

Binding of Isolated Natural Antigalactosyl IgG Antibodies to IgG-Depleted Thalassemic RBC

Thalassemic RBC were depleted of their autologous IgG by incubation with 0.15 M galactose for 30 min at 37°C (see Fig. 3). These IgG-depleted RBC were incubated with the isolated natural antigalactosyl IgG antibodies for 30 min at 24°C. Thereafter, the RBC were washed, incubated for additional 30 min with antoglobulin reagent, and assayed for EA rosetting with K-562 cells.

RESULTS

Demonstration of IgG Antibodies on the Membrane of Thalassemic RBC

The Ig bound to the RBC was assayed by the EA-DAT in 80 thalassemic patients. In 73 cases, the proportion of EA rosettes ranged between 5% and 100%, indicating the presence of Ig molecules on the RBC, whereas in 7 patients, the RBC formed less than 5% rosettes (Fig. 1). The RBC of 6 of the β-thalassemia intermedia patients formed EA rosettes as well as the RBC of the β-thalassemia major patients, whereas RBC from two patients formed no EA rosettes. No significant difference was found in the capacity of EA rosetting between RBC of splenectomized and nonsplenectomized patients. In 45 of 50 samples from normal donors, no rosette formation was observed, while in the remaining 5 samples the rosettes did not exceed 4%. When the assay was repeated using specific rabbit anti-human IgG or IgM, rosettes were found when thalassemic RBC were incubated with anti-IgG serum, whereas only two RBC samples rosetted following incubation with anti-IgM. The sera of the patients were simultaneously examined for alloantibody by EA-IDAT. In all but 2 sera, no activity was found (Fig. 1). These two sera were from the same patients whose RBC formed the rosettes following incubation with anti-IgM antibodies. Therefore, it
appears that the IgG bound to the RBC from the thalassemic patients is directed against autologous antigenic determinants. In 28 patients, the EA-DAT was performed on 2–4 separate occasions over a period of 4 mo. Three cases repeatedly exhibited no rosette formation. In one patient, the RBC formed 25% rosettes when initially assayed, but no rosettes could be detected when the assay was performed 2 mo later. The RBC of the remaining 23 cases always exhibited rosette formation in the range of 20%–100%. The EA rosetting assay was performed on 7 samples of thalassemic RBC that had been separated into different “age” populations. The “old” RBC formed 3–4-fold more EA rosettes than the “young” RBC (Fig. 2).

Analysis of the light chain type of the thalassemic RBC-bound IgG demonstrated a mixture of molecules bearing kappa and lambda chains (Table 1). The ratio of the bound light chain types differed from patient to patient. However, the coexpression of both types of light chains in each patient indicates polyclonality of the IgG-bound RBC.

Table 1. Analysis of Light Chain Type in IgG Molecules Bound to Thalassemic RBC

<table>
<thead>
<tr>
<th>Patient</th>
<th>Anti-Ig (Broad Spectrum)</th>
<th>Anti-Kappa</th>
<th>Anti-Lambda</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) M.O.</td>
<td>58</td>
<td>18</td>
<td>33</td>
</tr>
<tr>
<td>(2) G.O.</td>
<td>53</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>(3) A.S.</td>
<td>30</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>(4) I.D.</td>
<td>25</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>(5) S.A.</td>
<td>49</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>(6) B.H.</td>
<td>23</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>(7) G.S.</td>
<td>37</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>(8) H.C.</td>
<td>21</td>
<td>13</td>
<td>6</td>
</tr>
</tbody>
</table>

Results are expressed as percent of rosetting K-562 cells.

Specific Elution of the Thalassemic RBC-Bound IgG by Galactose

A mean decrease of 60% was found in the amount of EA rosettes formed by the RBC incubated with 0.15 M galactose (Fig. 3). No other carbohydrate tested decreased the capacity of thalassemic RBC to form EA rosettes. These findings suggest that the IgG found on the thalassemic RBC is directed mostly against terminal galactosyl residues exposed on these cells.

The specific inhibition of the rosette formation by galactose was found to be dose dependent, as indicated in Table 2. Nevertheless, no complete inhibition could be achieved even at the presence of the relatively high galactose concentration of 0.15 M.

Analysis of the IgG Eluted From the Thalassemic RBC

The reduced SA in the glycoprophin fraction of thalassemic RBC1-3 may result in the exposure of galactosyl residues, which are usually underlying the terminal SA.16-18 The exposed residues may serve as new antigenic sites and may bind autologous antigelactosyl IgG antibodies. To substantiate this assumption, the antibodies were eluted by heat and studied for their capacity to bind to RBC bearing terminal galactosyl residues. Rabbit RBC were used for these experiments, since they bear a large amount of terminal β-D-galactosyl residues as indicated by their distinct binding of the peanut agglutinin.19

RabRBC were incubated in eluates of thalassemic RBC bearing relatively high amounts of autologous IgG (>40% EA rosette formation). Incubation was performed at 24°C for 30 min. The RabRBC were washed and assayed for rosette formation with K-562 cells. Four of the supernatants from 14 cases studied
conferred sufficient concentrations of IgG to the RabRBC for the formation of 15%–37% rosettes. However, if anti-human serum was added prior to the assay, rosettes were formed in all the tested cases (Fig. 4). IgG specificity of the eluted molecules was shown by the fact that in 13 cases only anti-human IgG and not anti-human IgM enabled the formation of rosettes with K-562 cells. In one instance a low IgM activity was found in addition to a very high amount of IgG (Fig. 4). In order to examine whether IgG molecules bound to RabRBC could likewise be specifically removed, the eluate-treated RabRBC were incubated for 30 min at 37°C with 0.15 M of various carbohydrates, washed, incubated with anti-human serum, and assayed for rosette formation. Figure 5 shows that incubation with galactose resulted in an almost complete elution of the IgG molecules from the RabRBC. Incubation with glucose resulted in a partial removal of the IgG molecules, whereas all other carbohydrates tested had no significant effect on the formation of rosettes. Thus, the eluted IgG from thalassemic RBC seems to bind to RabRBC via the galactosyl residue.

Natural Antigalactosyl IgG Antibodies in Human Serum

Normal human adult sera regularly contain antigalactosyl IgM antibodies, reactive with the T antigen or

<table>
<thead>
<tr>
<th>Galactose Concentration:</th>
<th>0.15 M</th>
<th>0.075 M</th>
<th>0.037 M</th>
<th>0.018 M</th>
<th>0.008 M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent rosette formation</td>
<td>8.2 ± 1.2*</td>
<td>17 ± 1.6</td>
<td>21 ± 0.9</td>
<td>26 ± 3.0</td>
<td>33 ± 5.4</td>
<td>33 ± 3.1</td>
</tr>
<tr>
<td>Percent inhibition</td>
<td>75 ± 3.7</td>
<td>49 ± 4.8</td>
<td>36 ± 2.6</td>
<td>21 ± 8.7</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SE of data obtained from RBC of 6 patients with thalasemia.

Fig. 4. Binding of IgG eluted by heat from thalassemic RBC to rabbit RBC. Following incubation with the eluates, the rabbit RBC were washed and assayed for EA-DAT with anti-human serum or with specific anti-human IgG and anti-human IgM antibodies. Bar represents mean of results ± SE.

Fig. 5. Carbohydrate elution of rabbit RBC-bound IgG. The IgG assayed was previously eluted by heat from thalassemic RBC. Details as in Fig. 3.

Thomsen-Friedenreich antigen.20,21 In search for possible natural antigalactosyl IgG antibody, cord blood sera samples were used, since they lack the anti-T antibodies.22 IgM molecules are generally not found in cord blood, since unlike IgG, the IgM molecule does not cross the placenta. Following incubation with 1:10 dilution of all the 30 cord blood sera tested, the RabRBC exhibited a high capacity for EA rosetting (40%–95%). This direct rosetting further indicates the binding of IgG molecules, since the Fe portion of the IgG subclass, but not of the IgM molecules, can bind to the Fe receptor on the myeloid cell. The cord blood IgG molecules binding to RabRBC interacted with galac-
isolated from normal sara

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of antigalactosyl IgM activity (anti-T antibodies), or carbohydrates (Fig. 6). In 8 of 20 adult sera tested, a of the thalassemic RBC that were not depleted of the isohemagglutins.

The natural antigalactosyl antibodies were isolated from normal sera of 5 individuals with different blood groups.

tosyl residues, as shown by the almost complete elimination of rosetting capacity observed following incubation of the RBC with galactose, but not with other carbohydrates (Fig. 6). In 8 of 20 adult sera tested, a similar antigalactosyl IgG activity was detected utilizing the RabRBC. However, in 12 adult sera, excess of antigalactosyl IgM activity (anti-T antibodies), or antigalactosyl IgG subclasses unable to bind to Fc receptor of K-562 cells, caused clumping but no rosetting of the RabRBC and did not enable the analysis of the antigalactosyl IgG antibodies present in the sera. The natural antigalactosyl antibodies were isolated from 5 normal sera by absorption on RabRBC, elution with galactose, and removal of galactose by dialysis. These isolated antibodies could bind to thalassemic RBC that were formerly depleted of their autologous IgG by galactose elution (Table 3). In the 6 patients studied, the isolated natural antibodies restored the original rosetting capacity of the RBC as observed prior to incubation with galactose. Studies on control RBC with known phenotype showed no contamination of isohemagglutins. The isolated natural antigalactosyl IgG antibodies did not increase the rosetting capacity of the thalassemic RBC that were not depleted of the bound autologous IgG.

Antigalactosyl Ig Binding to Desialylated Human RBC

The demonstration of the natural antigalactosyl IgG antibodies prompted the investigation of the possible binding of these antibodies to the exposed galactosyl residues of autologous desialylated HuRBC as a result of neuraminidase treatment. Desialylated HuRBC were incubated with 1:10 dilutions of autologous serum, washed, and further incubated with antihuman serum. Following such procedure, the HuRBC readily formed EA rosettes. When prior to the addition of the rabbit anti-human serum, the RBC were incubated with 0.15 M galactose, the rosetting capacity was significantly decreased (Table 4). Similar results were obtained with the desialylated HuRBC when incubated with cord blood serum instead of autologous serum. Other carbohydrates exhibited no inhibiting effect on the rosette formation. These data indicate that following neuraminidase treatment, part of the immunoglobulins binding to the HuRBC interact specifically with the exposed galactosyl residues. Galactose did not affect the rosetting capacity of Rh’ HuRBC treated with anti-Rh antibodies.

**DISCUSSION**

Antoreactive IgG antibodies were demonstrated on RBC of 90% of patients with β-thalassemia major and in more than 50% of patients with β-thalassemia intermedia, utilizing the EA-DAT technique. These autoantibodies do not seem to be secondary to alloimmunization due to multiple transfusion, since only two patients had autoantibodies in the serum as indicated by EA-IDAT. In addition, the RBC of patients receiving few or no blood transfusions also exhibited surface-bound IgG within the same range as the transfused patients. Elution studies using various carbohydrates suggested that most of the RBC-bound IgG molecules interact specifically with galactosyl residues. The lack of complete inhibition of rosette formation even at the relatively high galactose concentration of 0.15 M can

<table>
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<tr>
<th>Table 3. Binding of Antigalactosyl IgG Antibodies Isolated From Normal Sera to IgG-Depleted Thalassemic RBC</th>
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<tbody>
<tr>
<td>Treatment of Thalassemic RBC</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>(1) IgG-bearing RBC</td>
</tr>
<tr>
<td>(2) IgG-depleted RBC*</td>
</tr>
<tr>
<td>(3) IgG-depleted RBC†</td>
</tr>
</tbody>
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*Results are expressed as mean ± SE of percent rosettes, from experiments in which the IgG-depleted RBC were incubated with natural antibodies isolated from normal sera of 5 individuals with different blood groups.

**Table 4. Elution of HuRBC<sub>don</sub> Bound Ig by Incubation With Various Carbohydrates as Assayed by EA Rosetting**

<table>
<thead>
<tr>
<th>Ig Source</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Fucose</th>
<th>Mannose</th>
<th>Arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous serum</td>
<td>48 ± 6.1†</td>
<td>17 ± 3.2</td>
<td>51 ± 6.5</td>
<td>47 ± 6.8</td>
<td>46 ± 8.5</td>
<td>49 ± 6.1</td>
</tr>
<tr>
<td>Cord blood serum</td>
<td>59 ± 4.7†</td>
<td>20 ± 4.3</td>
<td>54 ± 5.5</td>
<td>57 ± 7.1</td>
<td>48 ± 3.9</td>
<td>46 ± 8.2</td>
</tr>
<tr>
<td>Rh anti-Rh control</td>
<td>40</td>
<td>39</td>
<td>39</td>
<td>44</td>
<td>43</td>
<td>48</td>
</tr>
</tbody>
</table>

*Vibrio cholera neuraminidase-treated HuRBC.
†Mean ± SE of results obtained from 5 different individuals.
‡Assay performed on untreated Rh<sup>+</sup> HuRBC coated with anti-Rh antibodies.
be explained by the polyclonality of the antigalactosyl IgG antibodies. A portion of these antibodies may display a very high affinity for the galactosyl-containing antigen on the RBC. The IgG could also be efficiently eluted from the RBC by heating. The eluates obtained by thermal stripping of thalassemic RBC were used to confirm the antigalactosyl reactivity of the IgG. This was demonstrated by the specific binding of the eluted IgG to the free terminal galactosyl residues on RabRBC. The occurrence of IgG antibodies with a similar antigalactosyl reactivity could also be demonstrated in normal human serum by the interaction with RabRBC and with autologous desialylated RBC. Furthermore, natural antigalactosyl IgG antibodies isolated from normal sera could bind to thalassemic RBC that were formerly depleted of their autologous IgG by galactose elution. Therefore, it is possible that the accelerated removal of SA from thalassemic RBC results in exposure of the underlying galactosyl residues and the subsequent binding of the natural antigalactosyl IgG to "unmasked" previously cryptic antigenic sites.

Studies on the normal aging process of RBC demonstrated an age-related decrease in membranal SA concentration\(^7\) and concomitant binding of auto-IgG, as demonstrated by Kay.\(^6,9\) Alderman et al.\(^23,24\) have recently demonstrated immunoglobulins, mostly IgG, bound to membrane of old HuRBC. Following heat elution, these antibodies were found to bind to young desialylated HuRBC. The antigalactosyl IgG antibodies detected in the present study may be compatible with the auto-IgG antibody suggested by Kay\(^6,9\) and by Alderman et al.\(^23,24\) These antibodies possibly account for the selective removal of senescent cells by the reticuloendothelial system. In the thalassemic patient, the binding of these antibodies to the RBC with reduced and uneven surface distribution of SA\(^1\) may result in their shortened lifespan. The reason for the rapid removal of the SA from the thalassemic RBC is not clear as yet, and no increased sialidase activity has been detected in thalassemic sera.\(^3\)

The antigalactosyl IgG antibodies demonstrated in the present study do not seem to be part of the natural antibody moiety designated as polyagglutinins, or anti-T (Thomsen-Friedenreich) antibodies, since anti-T antibodies belong to the IgM subclass, are not found in cord blood, and do not react with autologous desialylated RBC.\(^20-22\) It is possible, however, that the anti-T antibodies and the antigalactosyl IgG antibodies are reactive against similar antigenic determinants. The in situ binding of the antigalactosyl IgG antibodies to the pathologic thalassemic RBC may reflect a general process involved in the physiologic aging of normal RBC and in amplified form in congenital and acquired hemolytic anemias characterized by reduced amount of SA on the RBC.\(^25\) The inhibition of the antigalactosyl antibody reactivity may offer a new therapeutic approach for prolongation of the lifespan of rapidly aging RBC.

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