Sickling-Induced Binding of Immunoglobulin to Sickle Erythrocytes

By Gloria A. Green and Vijay K. Kalra

Previously we demonstrated that sickle erythrocytes sedimenting at high densities after gradient centrifugation contain higher levels of surface immunoglobulin bound in vivo in comparison to low-density erythrocytes from the same patient. The present study examines the possibility that binding of autologous IgG to sickle erythrocytes may be associated with the sickling phenomenon. In the present study we subjected low-density erythrocytes to prolonged sickling under nitrogen in the presence of platelet-poor autologous plasma with added glucose for 24 hours (37°C). After reoxygenation IgG bound in vitro was quantified by a nonequilibrium autoradiated protein A-binding assay and by flow cytometry. Results show that sickle erythrocytes incubated under nitrogen bound significantly (P < .001) more IgG, 439 ± 41, molecules of IgG per cell (mean ± SD) compared with sickle cells incubated under oxygenation (227 ± 12 molecules of IgG per red cell) or compared with 196 ± 26 molecules IgG per cell for untreated sickle cells. In contrast, normal erythrocytes incubated in autologous plasma exhibited no detectable IgG binding in vitro under either oxygenation or deoxygenation. Flow cytometry shows that deoxygenation of sickle cells generated a two- to-sixfold increase in the subpopulation of brightly fluorescent IgG-positive cells in comparison to oxygenated sickle cells and a 13.5% ± 3.1% (mean ± SD) increase in median fluorescence intensity for fluorescein isothiocyanate–labeled deoxygenated sickled cells compared with labeled oxygenated sickle cells. Our studies demonstrate that prolonged sickling will induce in vitro binding of autologous IgG to sickle erythrocytes. These findings indicate that sickle erythrocytes may be unique when compared with erythrocytes from other nonimmunologic hemolytic anemias or senescent red cells in that the primary events producing surface antigens recognized by autoantibody may include the sickling process. These findings also suggest that sickling in vivo may generate membrane alterations in sickle erythrocytes that lead to cumulative binding of autoantibody in vivo.

We have initiated investigations of the mechanisms responsible for IgG binding to sickle RBCs. In the present study, we examined the possibility that binding of autologous IgG to sickle RBCs may be associated with the sickling process. To explore this possibility we have subjected low-density sickle RBCs to prolonged sickling by deoxygenation in vitro in an attempt to generate membrane alterations that could be analogous to membrane changes produced by a history of sickling. Results presented in this report demonstrate that sickle RBCs subjected to prolonged sickling in vitro bind significantly more autologous IgG compared with sickle cells incubated under oxygenation and in contrast to normal (HbA) red cells.

MATERIALS AND METHODS

Normal and sickle RBC fractions were prepared from whole blood specimens obtained from type O, Rh-positive donors by centrifugation on a discontinuous Stratrac II gradient as described previously. Sickle cell blood specimens (hemoglobin genotype, HbSS) from 21 adult patients were obtained from The Comprehensive Sickle Cell Center, USC–Los Angeles County Medical Center, after informed consent and approval of the University of Southern California Human Subjects Research Committee. Sickle cell patients who received blood transfusions within 0 to 4 months before the study were excluded from this investigation. Normal blood specimens (hemoglobin genotype, HbAA) were obtained from healthy volunteers. Specimens were screened for alloantibodies and depleted of leukocytes as described.

Incubation of erythrocytes with autologous plasma. Normal and sickle RBCs sedimenting at low specific density (<1.096 g/mL and >1.086 g/mL) were isolated as described and used in these studies. Reticulocyte-enriched sickle cells sedimenting at lower specific densities were removed from the top of the gradient by aspiration and discarded. RBC fractions were washed five times in 20 to 30 vol of phosphate-buffered saline (PBS), ph 7.4, containing 250 mg/L penicillin G, 300 mg/L streptomycin, and 16 mmol/L glucose (buffer A). The RBCs were suspended in autologous plasma that was made platelet poor by high-speed centrifugation (22,000 g for 30 minutes). Aliquots of a sterile concentrated solution contain-
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Measurements of fluorescence intensity. The frequency of cells exhibiting fluorescence and the relative intensity of the fluorescence were determined by using a Cytofluorograph 50H (Ortho Diagnostic Systems, Raritan, NJ) or Becton Dickinson (Mountain View, CA) equipped with an argon ion laser operated at a 488-nm wavelength. The frequency of cells exhibiting fluorescence was determined by FACS IV-interfaced computer and the relative intensity of fluorescence was computed by subtracting the background fluorescence intensity of test cells from the fluorescence intensity of control IgG-saturated, Rh-positive RBCs. Washed RBCs were incubated in buffer A containing 1.5% (wt/vol) bovine serum albumin (BSA; Sigma Chemical Co, St Louis, CA) for 24 hours with continuous purging by humidified nitrogen in a water bath shaker at 37°C. After incubation, the RBCs were resuspended in buffer A containing 3% (vol/vol) bovine serum albumin (BSA; Sigma Chemical Co, St Louis, CA) and incubated under conditions matched to the test cells. Specific binding of either radiolabeled protein A or fluorescein isothiocyanate (FITC)-labeled antimouse IgG by test cells was computed by subtracting the quantities bound by appropriate assay control RBCs. Assay control RBCs were also used for the experiments reported in Fig 2.

**RESULTS**

Because of our interest in determining whether prolonged sickling would induce binding of autologous IgG to sickle RBCs, we studied RBC subpopulations that contain low levels of surface IgG bound in vivo. In these studies, normal and sickle RBCs were subjected to prolonged deoxygenation under conditions that produce 97% to 100% sickling of RBCs and in the presence of sufficient glucose to maintain adenosine triphosphate levels. As shown in Table 1, sickle RBCs deoxygenated in plasma bound significantly more (P < .001) IgG compared with oxygenated RBCs: 439 ± 41 molecules IgG per RBC compared with 227 ± 12 (mean ± SD) for paired samples incubated under oxygenation or compared with preincubation IgG levels of 196 ± 26 molecules IgG per RBC. In contrast, normal (HbAA) RBCs exhibited no detectable in vitro binding of autologous IgG (Table 1).

The fluorescence intensity and frequency of brightly fluorescent IgG-positive cells generated during prolonged sickling was determined by immunofluorescence assays using flow cytometry for sickle RBCs incubated in autologous plasma. The median fluorescence intensity for FITC-labeled RBCs was 13.5% ± 0.02% (mean ± SD, n = 6) higher for deoxygenated sickle cells compared with oxygenated sickle RBCs incubated in plasma. The frequency of IgG-positive cells is compared for 13 different patient specimens (Table 2). The absolute fraction of IgG-positive cells varied among the 13 specimens studied (Table 2). However, analyses of paired samples from each patient (Table 2) demonstrate that prolonged deoxygenation produced a twofold or greater increase in the subpopulation of IgG-positive cells present before incubation and compared with RBCs oxygenated for 24 hours (Table 2). Five of the 13 patient specimens studied exhibited a detectable decrease in IgG-positive cells for RBCs incubated under oxygenation compared with untreated cells (Table 2). This decreased IgG binding could be due to dissociation of nonspecific cytophilic IgGs upon incubation at 37°C. However, in each of these five experiments deoxygenation generated a twofold or greater increase in the subpopulation of IgG-positive cells compared with either paired samples incubated under oxygenation or untreated RBCs (Table 2) as measured by flow cytometry.

Figure 1 shows the time course of IgG binding in vitro to sickle RBCs deoxygenated in autologous plasma. Results show essentially no difference in the quantities of IgG bound by deoxygenated or oxygenated sickle cells after eight to ten hours of incubation. However, sickle RBCs deoxygenated for 12 to 24 hours exhibit markedly increased IgG binding in comparison to oxygenated RBCs from the same patient (Fig 2).

<table>
<thead>
<tr>
<th>Table 1. Autologous IgG Bound to Normal and Sickle Erythrocytes After Prolonged Deoxygenation at 37°C</th>
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<tbody>
<tr>
<td>Molecules of 125I-Labeled Protein A Bound per Red Cell</td>
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<tr>
<td>Time = 0</td>
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<td>-----------------------------------------------</td>
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<tr>
<td>Sickle (HbSS) cells in plasma</td>
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<td>Normal (HbAA) cells in plasma</td>
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Numbers in parentheses indicate the number of different subjects studied. Data are reported as means ± SD. P < .001 for deoxygenated compared with oxygenated sickle RBCs.
In these experiments, deoxygenated sickle cells bound approximately fourfold more IgG in comparison to oxygenated RBCs after 24 hours incubation.

We have examined the possibility that RBC sickling in the absence of plasma may also generate binding sites recognized by autologous IgG. In these studies (Fig 2) sickle RBCs deoxygenated for 24 hours while suspended in buffer (assay control RBCs, see Materials and Methods) were subsequently incubated in autologous plasma. As shown in Fig 2, sickle RBCs preincubated at 37°C bind detectably more IgG in vitro in comparison to untreated RBCs, and sickle cells pretreated by deoxygenation bind more (approximately threefold) autologous IgG in vitro in comparison to oxygenated RBCs from the same patient (Fig 2).

DISCUSSION

The results presented in this report demonstrate that low-density sickle cells subjected to prolonged deoxygenation (under nitrogen) bind significantly ($P < .001$) more autologous IgG in vitro compared with oxygenated sickle cells (Table 1, Figs 1 and 2) and deoxygenated normal RBCs (Table 1). IgG binding assayed by flow cytometry showed that deoxygenation generated a two- to sixfold increase in the subpopulation of brightly fluorescent cells (Table 2) and a 13.5% ± 3.1% (mean ± SD) increase in median fluorescence intensity for deoxygenated cells compared with oxygenated sickle cells incubated in autologous plasma. These results indicate that the sickling-induced increase in the average number of IgG molecules bound per cell as determined by protein A binding (Table 1) is due both to the generation of new cell subpopulations binding large numbers of IgG molecules and to increased binding of comparatively small quantities of IgG by the major population of deoxygenated sickle cells. The results also show that increased IgG binding to sickle RBCs required 20 to 24 hours of deoxygenation (Fig 1), which suggests that new IgG binding sites result from some unknown time-dependent changes in the membrane surface associated with RBC sickling.

Although additional studies must be carried out to make conclusions regarding our observations, we postulate that new IgG binding sites result from RBC membrane alterations produced by the sickling process. Membrane alterations may involve both surface proteins or membrane lipids reorganized during the sickling process. It is possible that new IgG binding sites are produced by sickling-induced
reorganization of protein already present on the membrane surface. This protein reorganization could include (a) lateral translocation of integral proteins generated by their attachment to a membrane skeleton that may be distorted during the sickling process and (b) lateral translocation of other surface proteins resulting from mechanical distention of the membrane surface by growing hemoglobin (SS) fibers. Sickling-associated protein reorganization in the intact cell could produce clusters or aggregates of surface proteins that may then be recognized by autoantibody as suggested by studies on normal RBCs.

Previous studies have demonstrated that sickle RBCs sedimenting at a high specific density after gradient centrifugation contain excessive surface IgG bound in vivo in contrast to low-density sickle cells. Our present observations are consistent with the possibility that, because high-density sickle cells have been in the circulation for longer time periods relative to low-density sickle RBCs, excessive antibody binding could result from membrane alterations produced by the history of sickling-desickling cycles previously experienced by dense sickle cells. This hypothesis is supported by our demonstration that prolonged sickling in the absence of plasma (Fig 2) generates new antibody binding sites on sickle RBCs that could be detected even after reoxygenation. These observations also suggest that permanent sickling-induced membrane alterations resulting in antibody binding could be produced by a history of RBC sickling in vivo.

Our studies also suggest that sickle cells may be unique when compared with senescent RBCS or RBCs from other hemolytic anemias in that the primary events producing antigens recognized by autoantibody may include the sickling process. Studies are being carried out to further elucidate these possibilities.

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