Autologous IgM, IgA, and Complement Binding to Sickle Erythrocytes In Vivo: Evidence for the Existence of Dense Sickle Cell Subsets

By Gloria A. Green

We have previously reported that sickle erythrocytes sedimenting at high specific density after gradient centrifugation exhibit increased IgG binding in vivo as compared with low-density paired samples. We have performed the present study to determine whether the opsonization of dense sickle cells in vivo could also involve autologous IgM, IgA, and complement. IgA, IgM, and complement binding in vivo to the surface of density-separated sickle erythrocytes was detected by flow cytometric analyses. IgM and complement C3 fragment binding was detected primarily on high-density sickle erythrocytes. With the exception outlined below, IgA binding was detected for all sickle cell fractions that sediment at densities > 1.085 g/mL. IgM, IgA, and complement C3 fragment binding was increased on high-density sickle erythrocytes as compared with low-density paired samples and exceeded that binding to normal erythrocytes by 30% ± 10% (mean ± range), 50% ± 10%, and 41% ± 5%, respectively. Two-color flow cytometry indicates that high-density sickle cell fractions contain at least two heterogeneous RBC subsets. One is an RBC subset that binds IgA in combination with IgM and C3, and the second subset is devoid of IgA yet binds IgM and C3. These findings indicate that high-density sickle cells exhibit a greater heterogeneity than has been reported in previous studies, which is based on autologous Ig binding in vivo; and suggest that RBC components of this most severely dehydrated sickle cell subpopulation could have heterogeneous origin and pathophysiologic significance. Although the functional role of IgA binding to human RBCs is unclear, our findings that IgM and complement bind to the same high-density sickle cell fractions suggest that both the IgM and the sickle erythrocyte-bound IgG determined in previous studies could mediate the deposition of complement on dense sickle cells in vivo. These findings support the hypotheses that irreversibly sickled cell-enriched high-density sickle RBC subpopulations could be removed from the circulation by erythrocyte phagocytosis that is enhanced by the presence of complement.

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with the guidelines of the University of Southern California School of Medicine Committee on Human Subjects. Blood specimens used in this study were obtained from patients who were not in crises and had not received blood transfusions within the 3 months before this investigation. Blood was drawn into heparinized tubes.

Erythrocytes were isolated by centrifugation for 10 minutes at 2,000 rpm and then depleted of leukocytes by passage through cellulose columns as described. Sickle erythrocytes from six different DAT-negative homozygous (HbS) sickle hemoglobin specimens were density separated by centrifugation on a discontinuous sucrose gradient. Reticulocyte-rich sickle RBCs that sedimented at low density (<1.085 g/mL) were discarded. The remaining fractionated RBCs were washed six times in phosphate-buffered saline (PBS) containing 10 mmol/L HEPES (pH 7.4) at 25°C and then labeled as described below. The density-separated sickle RBC fractions 1, II, and III represent approximately 10%, 25%, and 35% of the total sickle RBCs remaining, respectively, after the least-dense reticulocyte-enriched fraction was removed.

Indirect immunofluorescence assays using flow cytometry. Washed normal adult hemoglobin (HbA) and HbS erythrocytes were subjected to mild fixation with 0.075% glutaraldehyde in PBS/HEPES buffer, washed in PBS, and then divided into aliquots. RBC aliquots were labeled with either biotin-conjugated antihuman IgA, IgG, or IgM or were labeled with antiserum to human complement C3, or albumin, or plasminogen as follows. Antihuman IgG binding was assayed in conjunction with these analyses to serve as a reference standard. Nonspecific binding sites on the fixed erythrocytes were first blocked by successive incubations with 2% bovine serum albumin (BSA) in PBS containing 0.02% sodium azide (buffer A) and fourfold diluted nonspecific serum. After two washes in buffer A, the cells were incubated for 16 hours (at 4°C) with saturating concentrations of either biotin-conjugated F(ab')2 fragments of antihuman IgA, IgM, or IgG (Sigma Chemical Co, St Louis, MO). After three washes with buffer A, the RBCs were labeled with streptavidin-phycocerythrin (Caltag, San Francisco, CA) for 1 hour at 4°C. For detection of complement C3 fragment binding to sickle erythrocytes, the RBCs with and without fixation were blocked as described above and then incubated with antihuman C3 fractionated sera (Sigma Chemical Co) that had been preabsorbed against aliquots of the normal HbA RBCs used as controls in this study. The RBCs were then stained with phycocerythrin (PE) and analyzed by flow cytometry as described below.

For the analyses of serum protein binding, aliquots of high-density sickle RBCs and normal erythrocytes were blocked and then labeled by 16 hours (at 4°C) of incubation with saturating concentrations of either goat antihuman serum albumin, or antihuman plasminogen (Sigma Chemical Co). After three washes with buffer A containing Tween 20 (0.001% vol/vol), the RBCs were labeled with biotin-conjugated goat IgG according to the instructions of the manufacturer (Sigma Chemical Co) and then labeled with streptavidin-phycocerythrin for 16 hours at 4°C. The fluorescence intensity and forward and right-angle light scatter for the labeled cells were measured as compared with appropriately labeled control cells using a FACSscan flow cytometer (Becton Dickinson, Richmond, CA) as described.

Two-color flow cytometric analyses of high-density sickle erythrocytes were performed essentially as described. Briefly, high-density sickle RBCs were fixed (0.075% glutaraldehyde in PBS), blocked, and labeled with biotin-conjugated F(ab')2 antihuman IgM or IgG or with antihuman C3, as described above. After washing in PBS, the RBCs were suspended in a 1:1 mixture of PE-conjugated streptavidin (diluted 1:4) and fluorescein isothiocyanate (FITC)-conjugated antihuman IgA-F(ab')2 (a-chain specific) that had been diluted 1:50 with 1.5% BSA in buffer A. The RBC mixtures were incubated for 16 hours at 4°C. The RBCs were washed in buffer A at 4°C and then analyzed by flow cytometry as outlined. The Ig-positive RBCs were defined in fluorescence-activated cell sorter (FACS)-generated contour diagrams taking into account the Ig-negative RBCs detected in controls labeled with nonimmune serum without subtracting background fluorescence as described. The net percentage of RBCs positive for two-color labels (ie, IgA and IgM or IgA and C3 fragments) (see Fig 3, Results section) were computed by subtracting the percentage of positive cells in quadrant II (see Fig 3) determined for high-density sickle RBC aliquots singly labeled with FITC-anti-human IgA or with either of the PE conjugates from the percentage of RBCs positive for both FITC and PE.

Other procedures. The IgA, IgM, and IgG content of the sera from specimens used in this study were assayed by radial immunodiffusion. In a separate set of assays, chloroform eluates were prepared from unfractionated sickle erythrocytes and/or normal RBCs previously washed six times in PBS containing 10 mmol/L HEPES (pH 7.4, at 25°C). The sickle cell eluates were tested for antibody activity at 37°C with an indirect antiglobulin test (IAT) against a commercial panel of normal RBCs with defined antigenic composition (Baxter/Dade Division, Florida); against high-density normal RBCs, and autologous dense sickle RBCs (with and without prior elution of surface Ig). In some studies, eluate-treated RBCs were further labeled sequentially with the polyclonal antiglobulin (used for the IAT) and PE-conjugated antirabbit IgG. The labeled RBCs were assayed by flow cytometry to determine Ig binding as compared with the appropriate controls including low-density normal RBCs.

RESULTS

Cell-bound autologous IgA and IgM and complement C3 fragments bound in vivo to sickle erythrocytes were detected by flow cytometry. The relative levels of IgA and complement binding were compared for normal (HbA) erythrocytes and density-separated sickle erythrocytes that were isolated and subjected to extensive washings in isotonic media (see Materials and Methods).

IgM and IgA binding to sickle erythrocytes. Erythrocytes labeled with biotin-conjugated F(ab')2-antihuman IgM and PE (see Materials and Methods) exhibited increased fluorescence intensity as compared with control RBCs treated with nonimmune sera, indicating the presence of surface IgM. The median fluorescence intensity (Fig 1; Table 1) was increased for high-density sickle cell fractions as compared with low-density paired samples (fractions I and II; Table 1). Comparison of the median fluorescence intensity ratios (HbS:HbA) for each density-separated sickle cell fraction (Table 1) shows that high-density sickle cell fractions (fraction III) had 30% to 40% more surface-bound IgM than normal RBCs. Dense sickle cell fractions also contained a significantly larger subpopulation of brightly fluorescent cells binding relatively large quantities of surface IgM (Table 1) as compared with the low-density paired samples.

Consistent with the observations for IgM binding, cell-bound IgA was increased for high-density sickle cells as compared with low-density sickle RBC fractions (Fig 1; Table 2). However, IgA binding detected for density-separated sickle cell fractions I to III (Table 2) was increased by 36% to 61% over normal controls. The dense sickle cell fractions
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Fig 1. Immune flow cytometry profiles for density-separated sickle erythrocytes stained with antihuman IgM (top section) or antihuman IgA (lower section). Erythrocyte fractions isolated from a representative HbS patient specimen were labeled with PE and the log fluorescence intensity determined (see Materials and Methods). The fluorescence profiles indicated by broken lines (---) represent the low-density sickle erythrocyte fraction; the profiles indicated by the dotted lines (-----) represent the high-density sickle cell fraction (density > 1.1 g/mL).

(density >1.1 g/mL) also contained a two-fold larger RBC subpopulation than low-density paired samples (Table 2) that was sensitized with greater quantities of IgA.

The results listed in Tables 1 and 2 and Fig 1 show that both IgM and IgA are present on the same high-density sickle erythrocyte fraction, because aliquots of these density-separated fractions were separately labeled with either antihuman IgM or IgA. These data (Tables 1 and 2) also show that significantly larger proportions of the high-density sickle erythrocyte fractions bind greater relative quantities of IgA in vivo (≈ 25% ± 5% [mean ± SD]) as compared with IgM (≈ 7% ± 2%). It is important to note that the density-separated sickle erythrocytes labeled with either antihuman IgM or IgA in Fig 1 were obtained from a single patient specimen that yielded fluorescence profiles intermediate to the total (n = 6) specimens studied (Tables 1 and 2).

Ancillary studies using both dense normal and sickle RBC fractions that had been shown previously to contain surface Ig\(^{ii,i4}\) (Tables 1 and 2) were performed to examine the antibody activity of the sickle RBC-bound Ig. In the first set of assays, dense sickle and normal RBC fractions assessed by the DAT were unreactive. In a second set of assays, chloroform eluates derived from DAT-negative unfractionated sickle cells were tested against dense (>1.1 g/mL) normal RBCs, dense autologous sickle RBCs, and a commercial RBC panel with known antigen configurations using the IAT. The sickle cell eluates yielded weak agglutinations of dense autologous RBCs and dense normal RBCs using the IAT. Thirty-five percent to 40% of the eluates assayed showed reactivity with the entire panel of reagent RBCs.

Table 1. Autologous IgM Binding to Sickle Erythrocyte Fractions as Measured by Flow Cytometry

<table>
<thead>
<tr>
<th>Sickle RBC Fractions</th>
<th>Median Fluorescence Intensity</th>
<th>Ratio (HbS:HbA)</th>
<th>% RBCs Exhibiting High Relative Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density &lt;1.095 g/mL</td>
<td>139 ± 2 1 0.1 0.8 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Density &lt;1.1 g/mL</td>
<td>139 ± 3 1 0.1 0.8 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Density &gt;1.1 g/mL</td>
<td>180 ± 6 1.3 ± 0.1 7 ± 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The log fluorescence intensity in arbitrary units is reported as mean ± range for sickle erythrocytes labeled with biotin-conjugated anti-human IgM and PE (see Materials and Methods). The ratio (HbS:HbA) is the ratio of the average median fluorescence intensity for the designated HbS fractions:median fluorescence for unfractionated normal control RBCs, mean ± range for n = 6 HbS specimens. Percent fluorescent RBCs reported as mean ± SD.
gated antirabbit IgG and then assayed by flow cytometry. Dense sickle RBCs pretreated at 56°C to elute most of the Ig bound in vivo were equilibrated with autologous sickle RBC eluates and then incubated with the polyspecific antisera used for the IAT described above. The RBCs were labeled with PE-conjugated antihuman IgA and PE (see Materials and Methods). The data units are the same as described in Table 1 for n = 6 HbS specimens.

The log fluorescence intensity is reported for sickle erythrocyte fractions labeled with biotin-conjugated antihuman IgA and PE (see Materials and Methods). The data units are the same as described in Table 1 for n = 6 HbS specimens.

Table 2. Autologous IgA Binding to Sickle Erythrocyte Fractions as Measured by Flow Cytometry

<table>
<thead>
<tr>
<th>Sickle RBC Fractions</th>
<th>Median Fluorescence Intensity Mean ± Range</th>
<th>Ratio (HbS:HbA)</th>
<th>% RBCs Exhibiting High Relative Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density &lt;1.095 g/mL</td>
<td>198 ± 4</td>
<td>1.36 ± 0.06</td>
<td>11.5 ± 0.5</td>
</tr>
<tr>
<td>Density &lt;1.1 g/mL</td>
<td>204 ± 2</td>
<td>1.42 ± 0.02</td>
<td>11.7 ± 0.5</td>
</tr>
<tr>
<td>Density &gt;1.1 g/mL</td>
<td>235 ± 3</td>
<td>1.61 ± 0.1</td>
<td>25 ± 5</td>
</tr>
</tbody>
</table>

The log fluorescence intensity is reported for sickle erythrocyte fractions labeled with biotin-conjugated antihuman IgA and PE (see Materials and Methods). The data units are the same as described in Table 1 for n = 6 HbS specimens.

consistent with previous reports by Petz et al. Because only weak reactions were obtained in the IAT using dense autologous RBCs, the reactivity of the sickle cell eluates was further assessed by flow cytometry. Dense sickle RBCs pretreated at 56°C to elute most of the Ig bound in vivo were equilibrated with autologous sickle RBC eluates and then incubated with the polyspecific antisera used for the IAT described above. The RBCs were labeled with PE-conjugated antirabbit IgG and then assayed by flow cytometry. These assays showed that dense sickle RBCs treated with autologous RBC eluate bind more polyspecific antisera than control RBCs as indicated by both (1) the consistent increases in mean fluorescence intensity for all specimens studied, and (2) the increase in the subpopulation of brightly fluorescent RBCs observed for three of four HbS specimens. These results indicate that the Ig eluted from sickle RBCs will bind to autologous dense RBC subpopulations. Control assays also showed that dense sickle RBCs pretreated at 56°C did not exhibit nonspecific protein binding and that 56°C-treated low-density normal RBCs did not bind autologous RBC eluates. Although final conclusions require additional studies, the results from this series of sensitive fluorescence assays suggest that autoantibody activity1,20 is present in sickle RBC eluates.

Serum levels of IgA, IgG, and IgM for the specimens used in this investigation were also analyzed by radial immunodiffusion (data not shown) and found to contain Ig concentrations consistent with previous reports.15

Complement C3 fragment binding to sickle erythrocytes. Because both erythrocyte-bound IgM and IgG have the capacity to fix complement, we next examined the possibility that complement C3 fragments may be deposited on sickle erythrocytes in vivo. Antihuman C3 binding to high-density sickle cell subpopulations was increased over that binding to low-density paired samples, as indicated by the rightward shift in fluorescence profiles (Fig 2), and the increased median fluorescence intensities listed in Table 3. Increased C3 binding was apparent for dense sickle RBCs labeled with antihuman C3 before or after mild fixation with glutaraldehyde. C3 binding to high-density sickle cells was increased by 41% ± 5% (mean ± range) over normal RBCs (Table 3).

Analogous to the results obtained for IgM or IgA binding (Tables 1 and 2), a subpopulation of brightly fluorescent erythrocytes stained with antihuman C3 was present in each sickle cell fraction (Table 3). This population was consistently greater in high-density sickle RBC fractions as compared with the low-density paired samples (Table 3). The fluorescence profiles comparing anti-human complement C3 binding by high- and low-density sickle cell fractions (Fig 2) were prepared using aliquots of the same HbS specimen illustrated in Fig 1. Although the fractionated anticomplement serum used in the study does not qualitatively distinguish between bound C3 fragments, these results (Table 3; Figs 1 and 2) do show that complement C3 fragment binding can be detected for the same high-density sickle cell fractions that exhibited both increased IgM and IgA binding in vivo (Tables 1 and 2).

![Fig 2. Immune flow cytometry profiles for density-separated sickle erythrocytes binding antisera to human complement C3 fragments. Density-separated sickle erythrocytes isolated from the representative HbS specimen illustrated in Fig 1 were labeled with antisera to human complement C3 fragments and PE and the log fluorescence intensity measured (see Materials and Methods). The fluorescence profile indicated by broken lines (-----) represents the low-density sickle cell fraction; the profile indicated by dotted lines (· · · · · ·) represents the high-density sickle RBC fraction sedimenting at densities > 1.1 g/mL after gradient centrifugation.](image-url)
LG AND COMPLEMENT BINDING TO SICKLE RBCs

Two-color flow cytometric analysis of high-density sickle erythrocytes. Because the immunofluorescence assays using single PE labeling showed that high-density sickle erythrocytes bind more IgM, IgA, and C3 fragments than low-density paired samples or normal erythrocytes (Figs 1 and 2; Tables 1, 2, and 3), we subsequently performed two-color flow cytometric analyses of high-density sickle RBCs to determine the frequency of cells within this population that binds IgA in combination with IgM or C3 fragments. Figure 3 shows FACS-generated contour plots for a representative specimen. Analysis of these data show that the dense sickle erythrocyte subpopulation contains a subset of RBCs (5.1%, corrected for background fluorescence) that binds both IgA and IgM (Fig 3D) and a 41.5% RBC subset that binds both IgA and C3 fragments (Fig 3F). Contour plots gated on the same coordinates for the singly labeled controls show that 28.4% (Fig 3C) of the dense sickle cells were labeled with antihuman IgM, 98.1% of these cells were stained by anti-human C3 (Fig 3E), and 76% of these RBCs stained with FITC-anti-human IgA-F(ab)2 (Fig 3B). These analyses show that although most of the dense sickle cells bind IgA and C3 in vivo, at least 23.3% of the cells binding IgM were devoid of IgA. These results also indicate that this subset of dense sickle erythrocytes (~23.3%) binds C3 in combination with IgM.

Non-specific binding of serum proteins to high-density sickle erythrocytes. Because several studies have suggested that high-density sickle cells exhibit changes in the membrane surface that increase the adhesivity of sickle RBCs, it is possible that this adhesivity or relative "stickiness" could be reflected by an increased tendency to bind serum proteins, including IgGs. We therefore measured the relative binding in vivo of irrelevant serum proteins, including serum albumin, and plasminogen, by high-density sickle cells as compared with normal erythrocytes. The ratio of mean fluorescence intensities for normal:high-density sickle RBCs labeled with anti-human serum albumin or anti-human plasminogen and PE were 1.04 ± 0.06 and 1.10 ± 0.08 (mean ± SD; n = 4 different HbsS specimens), respectively. These results show that normal RBCs bind slightly larger quantities of serum albumin, or plasminogen in vivo than high-density sickle erythrocytes. Whereas in contrast, the results reported in Tables 1, 2, and 3 show that high-density sickle RBCs bind significantly larger quantities of IgM, IgA, and complement C3 in vivo as compared with normal erythrocytes.

DISCUSSION

This study was performed to determine whether autologous IgA, IgM, or complement bound in vivo could be detected on sickle erythrocytes. This possibility was explored because previous studies have shown that sickle erythrocytes bind autologous IgG in vivo; because opsonization of dense sickle RBCs was suggested by the finding that sickle RBCs can be phagocytized by macrophages in vitro; and because serum from patients with sickle cell anemia contained increased quantities of IgG and IgA and variably increased levels of IgM. The present study shows that IgA, IgM, and complement C3 fragments bound in vivo can be detected on sickle erythrocytes by flow cytometry. The relative levels of IgM, IgA, and C3 fragments were increased on sickle erythrocytes that sediment at high specific density after gradient centrifugation as compared with normal (HbA) erythrocytes (Tables 1, 2, and 3) or low-density sickle cell fractions (Figs 1 and 2) consistent with previous studies on IgG binding. Our study shows that high-density sickle RBCs do not bind larger quantities of irrelevant serum proteins in vivo than normal RBCs (including serum albumin or plasminogen); therefore a significant proportion of the cell-bound IgGs detected (Tables 1 and 2; Figs 1, 2, and 3) could have some relative selectivity for dense sickle erythrocytes. Our observations that suggest that autoantibody activity eluted from sickle RBCs will preferentially bind to dense sickle RBCs also support these conclusions.

The presence of three major Ig classes (ie, IgA, IgM, and IgG) bound to dense sickle erythrocytes is analogous to previous observations for senescent normal erythrocytes. High-density normal RBCs that represented 2% to 5% of the total RBCs bound more IgG, IgM, and IgA in vivo than low-density normal RBCs. However, the present study shows that significantly larger numbers of sickle erythrocytes are sensitized by these IgGs because high-density sickle RBCs represent 22% to 38% of the total RBC population. These findings suggest that membrane features associated with RBC aging in vivo and relected by Ig-binding are exaggerated in dense sickle cells. However, a number of marked changes in morphology and membrane surface characteristics develop on dense sickle cells in vivo that unlike normal RBCs are directly related to the presence of Hbs. It is possible that membrane surface changes on dense sickle cells may result in the development of both specific and non-specific binding sites recognized by autologous IgGs. Our observation that high-density sickle RBCs do not bind more irrelevant serum protein than normal RBCs suggests that a significant fraction of the IgG binding determined for high-density sickle RBCs (Tables 1 and 2; Figs 1 and 2) involves some relative specificity.

Although it is not clear why IgGs bind to sickle erythrocytes in vivo, the increased IgA binding to a majority of the sickle RBCs could reflect the increased concentrations of IgA pres-
ent in the serum of patients with sickle cell anemia. Malave et al. showed that even nonstimulated peripheral blood mononuclear cells from patients with sickle cell disease will spontaneously synthesize more IgA and IgG in culture than normal controls. These observations may account for the increased IgG and IgA serum levels reported previously. Our findings that IgA binds to a significant fraction of RBCs present in each of the density-separated sickle cell subpopulations (Table 2) could indicate cyto-philic characteristics for a major proportion of the serum IgA that are not apparent in clinically normal individuals (ie, patients who have not experienced the history of infections peculiar to sickle cell anemia). However, some specificity is suggested by our observations that the IgA bound to dense sickle cell fractions (Table 2) is increased over low-density paired samples despite the relatively high plasma IgA concentrations. Serum IgM levels in patients with sickle cell disease were not consistently increased over normal controls; therefore, the increased IgM binding to high-density sickle erythrocytes (Table 1) could represent specific binding site recognition.

IgM, IgA, and complement C3 binding to high-density sickle erythrocytes was compared with aliquots of the same RBC fraction singly labeled with antihuman IgM and PE (D) or antihuman C3 and PE (F) and either antihuman IgA F(ab')2 and FITC-antihuman IgM F(ab')2 (C), antihuman C3 and PE (E), or by FITC-antihuman IgA (B). Analysis gates were set using the appropriate label controls (A). Erythrocytes positive for both IgA and IgM (D) or IgA and C3 (F) are indicated in quadrant II.

Fig 3. Two-color flow cytometric analyses of high-density sickle erythrocytes. FACS-generated contour plots showing log fluorescence intensity for high-density sickle erythrocytes dual-labeled (see Materials and Methods) with FITC-antihuman IgA F(ab')2 and either antihuman IgM and PE (D) or antihuman C3 and PE (F) are compared with aliquots of the same RBC fraction singly labeled with antihuman IgM and PE (C), antihuman C3 and PE (E), or by FITC-antihuman IgA (B). Analysis gates were set using the appropriate label controls (A). Erythrocytes positive for both IgA and IgM (D) or IgA and C3 (F) are indicated in quadrant II.
sickle erythrocytes was also studied by two-color flow cytometric analyses. These studies show that heterogeneous RBC subsets are present within the dense sickle erythrocyte fraction (Fig 3). Most of the high-density sickle RBCs bind both IgA and complement C3 fragments; however, small subsets could be detected that bind IgA in combination with IgM and C3 fragments. A small but significant RBC subset devoid of IgA yet binding IgM and complement C3 was also detected by two-color analyses. These findings indicate that along with the increased IgG binding determined previously, dense sickle cell fractions exhibit heterogeneity based on the binding of combinations of IgM or IgA with complement. These observations represent the first demonstration that high-density sickle cells exhibit heterogeneity based on Ig binding in vivo and indicate that dense sickle cells exhibit a greater heterogeneity than has been previously reported.

The presence of heterogeneous RBC subsets in dense sickle cell fractions (Fig 3) suggests that subsets of sickle cells develop unique changes in the membrane surface that could be reflected by the binding of either IgA or IgM in vivo. These observations are also consistent with the proposal that the components of dense sickle cell subpopulations may have heterogeneous origins. We hypothesize that the transformation of distinct sickle RBC subsets into dense sickle cells (ie, irreversibly sickled cells [ISCs] and/or non-ISCs) in vivo is accompanied by the development of distinct changes in membrane structure that generate the heterogeneous Ig-binding dense sickle cell subsets. Specifically, we have proposed that sickling-associated membrane remodeling may alter the surface distribution of integral membrane proteins creating new binding sites recognized by autologous Ig. Similar conclusions have been proposed in subsequent studies. It is possible that these new binding sites could be responsible for the increased binding of IgA to dense sickle RBCs reported here (Table 2; Figs 1 and 3) and the dense sickle cell–bound IgG determined previously, although the IgM may recognize the li epitopes present on sickle cells. Studies underway in our laboratory will further delineate the differences between high-density sickle RBC subsets.

The apparent parallel between the Ig-binding pattern (Tables 1 and 2) and the binding of antihuman complement C3 to dense sickle RBCs (Table 3; Figs 2 and 3) suggests that the cell-bound IgGs could influence the deposition of complement on sickle erythrocytes in vivo. It is possible that both the cell-bound IgM (Table 1; Fig 1) and the IgG detected previously could mediate complement binding to sickle RBCs, because it has been established that both antigen-bound IgM and IgG fix complement. However, the studies by Lutz et al have suggested that anti-band 3 IgG-mediated deposition of complement on the surface of oxidant-treated or senescent normal RBCs results from the activation of the alternative complement pathway. Although the functional interaction of human Ig A with complement appears controversial, some evidence has been reported that suggests IgA could activate the alternative complement pathway. Our current finding that IgA binding to dense sickle RBCs is increased (Table 2; Fig 1) suggests that complement deposition on dense sickle cells could be produced by similar mechanisms mediated by IgA and/or IgG. Although elucidation of the complement-binding mechanism is beyond the scope of the current study, our report provides evidence that complement C3 fragments in combination with IgM are selectively bound to a subset of high-density sickle erythrocytes, which suggests that a subset of dense sickle RBCs may be opsonized in vivo by the classical mechanism.

Previous studies show that the number of IgG molecules binding to sickle cells is variable among patient specimens and often below the threshold required to promote phagocytosis by macrophages; therefore, our findings that multiple Igs as well as complement bind to dense sickle cells in vivo suggest that this RBC subpopulation could be subject to complement-enhanced phagocytosis in vivo. Moreover, these observations could explain the previous studies on tissue taken at autopsy that showed that sickle cells were present in the Kupffer cells of the liver of functionally asplenic patients with sickle cell anemia. Our observations that complement binds to sickle cells in vivo are also consistent with the recent suggestion that sickle RBCs could be more sensitive to complement-mediated hemolysis because of the loss of inhibitor proteins through membrane vesiculation.

In summary, our study shows that dense sickle cell subpopulations bind more IgA, IgM, and complement C3 in vivo than low-density paired samples or normal erythrocytes and exhibit a greater heterogeneity than previously reported, which is based on autologous Ig binding in vivo. Our analyses demonstrate that high-density sickle cell fractions contain at least two heterogeneous Ig-binding RBC subsets that bind either IgA and IgM in combination with C3; or IgM and C3. These findings further support the hypothesis that sickle erythrocytes, at least discrete dense sickle RBC subsets, may be removed from the circulation by phagocytosis, because erythrocyte phagocytosis by macrophages is enhanced by the presence of both Ig and complement.

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