Phagocytosis of Sickle Erythrocytes: Immunologic and Oxidative Determinants of Hemolytic Anemia

By Robert P. Hebbel and Wesley J. Miller

Hemolytic anemia in sickle disease involves both intravascular and extravascular destruction of erythrocytes. Since the latter presumably involves the reticuloendothelial system, we have examined interactions between sickle erythrocytes and macrophages. In erythrophagocytosis assays, 18.9 ± 7.2% of human marrow macrophages ingest sickle RBCs, while only 3.1 ± 2.1% ingest normal RBCs. This abnormality is not explained by reticulocytosis, and it is strongly dependent upon RBC density. The interaction between sickle RBCs and macrophages appears to be partly immunologic, since it is partially blocked by Fc receptor blockade. Also, admixture of sickle RBCs (pre-treated with rabbit anti-human-Ig) and Fc-receptor-bearing K562 cells results in 15.8 ± 10.6% K562-RBC rosette formation compared with only 0.5 ± 1.2% for normal RBCs. Interestingly, abnormal in vitro interactions with sickle erythrocytes, sickle RBCs are found to spontaneously generate twice-normal amounts of dialdehyde byproducts of lipid peroxidation ("malondialdehyde" or MDA). Peroxide or reagent-MDA treatment of normal RBCs significantly enhances their phagocytosis, and MDA is at least 50 times more potent than other aldehydes studied here. Oxidative and immunologic effects may be related, since exposure of MDA-treated RBCs to immunoglobulin-containing human sera results in a further significant enhancement of erythrophagocytosis. For comparison of different sickle patients, an adherence assay demonstrates that sickle RBCs are 1.03 to 6.85 times more adherent to macrophages than are normal RBCs, and degree of adherence correlates significantly with irreversibly sickled cell (ISC) counts and hematologic variables reflecting hemolytic rate. We conclude that propensity for RBC interaction with macrophages is likely to be a determinant of hemolytic rate in sickle disease. Pertinent mechanisms appear to involve modification of RBC membranes by dialdehyde byproducts of excessive autoxidation and the abnormal acquisition of surface immunoglobulin on sickle RBCs, although participation of other membrane defects has not been excluded. Interestingly, the data further suggest the possibility that appearance of the "senescence antigen" in old normal RBCs represents modification of the membrane by "MDA."

MATERIALS AND METHODS

Erythrocytes

Fresh citrated blood was obtained from normal volunteers and folate-replete sickle cell anemia patients who were not acutely ill. Some had been transfused in the past but had no detectable RBC antibodies during extensive screening by our institution's blood bank. Additional control RBCs were obtained from patients with posthemorrhagic reticulocytosis and from anemic patients identified by the blood bank as having a positive direct Coombs test for IgG. The RBCs were washed three times with isotonic NaCl, with removal of the buffy coat after each wash. For some studies, washed RBCs underwent additional manipulations. Some RBCs were separated into subpopulations based on RBC density by centrifugation through a discontinuous density gradient system using dextran or a continuous gradient using Percoll (Pharmacia Fine Chemicals, Piscataway, NJ). Completed gradient

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separations were divided into five subpopulations of approximately equal size: the top, middle, and bottom fractions were studied. Some RBCs were heated to 47°C using described methods to elute any bound Ig.

Some RBCs were incubated at hematocrit 10% for two hours at 37°C in Hanks' balanced salt solution (HBSS) with various concentrations of malondialdehyde (MDA) at pH 7.4, prepared immediately before use by acid hydrolysis of malondialdehyde bis-(dimethyl acetal). Control RBCs were incubated with appropriate concentrations of methanol (CH3OH), since MDA preparation yields both CH3OH and MDA in a 4:1 molar ratio. As alternatives to reagent-MDA exposure, some RBCs were incubated at Hct 20% for two hours at 37°C in HBSS containing 10 mmol/L H2O2, and others were stored for 24 hours at 37°C in autologous plasma. Some RBCs were incubated at Hct 20% for two hours at 37°C in HBSS with or without 1 mmol/L glutaraldehyde, 1 mmol/L formaldehyde, 1 mmol/L carbodiimide (pH 7.4).

Some RBCs were exposed to Ig by incubation for 30 minutes at room temperature in serum derived from untransfused type AB/Rh+ normal male blood donors.

Some RBCs were labeled with 51Cr by incubation of 300 μL RBCs for 40 minutes at 37°C with 10 μCi 51Cr (Amersham Corp, Arlington Heights, Ill).

Preparations of manipulated RBCs were washed five times with saline before application to subsequent assays.

**Macrophage Studies**

**Macrophages.** Most studies employed normal human marrow macrophages, obtained from marrow transplant donors. Using established methods, fresh marrow aspirate was layered onto Ficoll-Hypaque and centrifuged at 400 g for 25 minutes. The interface mononuclear cells were washed and suspended to 2 x 10⁶ cells per milliliter in culture medium (CMRL 1066 [Grand Island Biological Co, Grand Island NY] supplemented with 15% fetal calf serum [Rehuis Chemical Co, Kankakee, Ill], 100 U/mL penicillin-streptomycin, and 2 mmol/L glutamine). One milliliter of cell suspension was placed within the chamber of a modified Marbrook flask with 75 mL culture medium in the outer portion of the flask. After ten to 14 days in liquid culture (at 37°C in air/5% CO2), approximately 70% of the viable cells morphologically resemble macrophages, are in a resting (unstimulated) state, and manifest macrophage characteristics such as phagocytosis, Fc receptor, adherence, and chemotaxis.

Limited experiments utilized human alveolar or splenic macrophages, obtained from bronchial lavage of nonsmokers and from surgical waste, respectively. Splenic tissue was first passed through fine wire mesh. Alveolar macrophages and splenic cell suspensions were centrifuged through Ficoll-Hypaque, and the interface mononuclear cells were incubated on plastic dishes for one hour at 37°C. After washing the plates with culture medium, adherent cells were mechanically resuspended in culture medium and used for experiments on the same day they were obtained.

Before their use for adherence experiments, macrophages were removed from liquid culture, allowed to adhere to the bottom of plastic culture wells, and washed three times with HBSS/Alb (Hanks' balanced salt solution with 0.5% human albumin). For phagocytosis assays, macrophages were left in the liquid culture system.

**Erythrocyte Adherence to Macrophages.** Quadruplicate aliquots of 250 μL 51Cr-labeled RBC suspension (Hct 25% in HBSS/Alb) were added to 16-mm plastic culture wells containing 5 x 10⁹ previously adhered macrophages. After 30 minutes incubation at 37°C, macrophage layers were washed six times with 300 μL HBSS/Alb. The remaining adherent RBCs were then lysed with two distilled water washes, and the percentage of RBCs remaining adherent was determined from the radioactivity in the wash volumes. Normal and sickle RBCs were examined in parallel, and sickle RBC adherence is expressed as a "macrophage adherence ratio" (percentage of sickle RBCs adherent divided by percentage of normal RBCs adherent).

**Erythrocyte Phagocytosis by Macrophages.** One-hundred microliters of RBC suspension (Hct 10% in saline) was added to 10⁶ macrophages suspended in 1 mL culture medium (target/effecter ratio of 100:1). After the mixture was incubated in air/5% CO2 for 15 hours at 37°C, it was centrifuged and the pellet exposed briefly to water to lyse uningested RBCs. Cytospin slides were stained with Wright-Giemsa stain, and at least 200 cells were examined to determine the percentage of macrophages ingesting RBCs.

**K562 Rosetting Studies**

To indirectly demonstrate surface-bound Ig, we searched for K562-RBC rosettes in mixtures of extensively washed RBCs and (Fc-receptor-bearing) K562 cells. Two-hundred microliters of washed RBC suspension (Hct 5% in saline) was centrifuged, and the pellet was resuspended in 0.2 mL antiserum (rabbit anti-human-Ig; Cappel Laboratories, Cochranville, Pa) and incubated at room temperature for 30 minutes. The RBCs were then washed five times with saline, and the pellet was adsorbed with 0.2 mL K562 cell suspension (2 x 10⁶ cells per milliliter in saline). The mixture was centrifuged for five minutes at 500 g and incubated for 60 minutes at room temperature. After resuspension of the pellet, the percentage of K562-RBC rosettes was determined by microscopic examination of wet mounts. As previously described, a rosette was defined as a K562 cell binding at least five RBCs. Each experiment used both a negative control (omission of the antiserum step) and a positive control (commercial Combs-positive RBC [D+/anti-D; Cooper Diagnostics Inc, Garden Grove, Conn]).

**Inhibition Studies**

Selected experiments were also performed in such a manner as to block Fc receptors of macrophages or K562 cells. The IgG for this purpose was prepared from normal human plasma passed over a Sepharose-Protein A column (Pharmacia, Piscataway, NJ). The bound IgG was eluted with glycine-HCl buffer (pH 2.8), dialyzed against water, lyophilized, and dissolved immediately before use to 10 mg/mL or 50 mg/mL in saline (pH 7.0). Fc or Fab fragments of human IgG (Cappel) were dissolved to 10 mg/mL in saline (pH 7.0).

For Fc receptor blockade experiments, 0.1 mL solution of IgG or Fc or Fab fragments was added directly to 0.9 mL macrophages in liquid culture, yielding final concentrations of 1 or 5 mg/mL IgG and 1 mg/mL Fc or Fab fragments. K562 cells were suspended to 2 x 10⁶ cells per milliliter in saline containing 1 mg/mL IgG. Phagocytosis or rosetting assays were then done as described above. The efficacy of our IgG preparation for blockade of Fc receptors was independently evaluated by examining its ability to inhibit formation of rosettes between normal human marrow macrophages and IgG-coated sheep RBCs. One milligram per milliliter IgG inhibited EA rosette formation by 54.0 ± 6.5% (n = 3), and 5 mg/mL inhibited rosette formation by 64.3 ± 6.5% (n = 5).

Other experiments examined the ability of various sugars (glucose, galactose, ribose, fructose, mannose, N-acetylgalactosamine, N-acetylgalactosamine, N-acetylgalactosamine, N-acetylgalactosamine, N-acetylgalactosamine, N-acetylgalactosamine, N-acetylgalactosamine, N-acetylgalactosamine) or amines (serine, ethanolamine, choline) to inhibit phagocytosis of sickle RBCs. Each substance was prepared at 0.5 mol/L in HBSS with pH adjusted to 7.0. The osmotic control substance was 0.25 mol/L NaCl. One-twentieth volume of potential inhibitor was added to macrophages in liquid culture, yielding a final concentration of 25 mmol/L. Erythrocytes were then added as above.
K562 studies, RBCs were separated using Percoll and a continuous density gradient. The top, middle, and bottom 20% selected for study.

In an attempt to block the phagocytosis-promoting effect of serum upon MDA-treated RBCs, 25 μL MDA-treated RBCs were incubated for 30 minutes at room temperature with 150 μL human IgG Fab fragments (10 mg/mL in saline; Cappel). After three saline washes, they were incubated with human serum, washed again, and assayed for phagocytosis as described above.

**Endothelial Studies**

Erythrocytes studied for adherence to macrophages were also examined for adherence to confluent, cultured human umbilical vein endothelial cells as previously described in detail.8

**Malondialdehyde Generation**

After filtration through cellulose, RBCs were incubated in HBSS with 1 g/L glucose at Hct 2.5% for 20 hours at 37°C. Aliquots were precipitated with trichloracetic acid, and MDA generated during the incubation period was measured using the thiobarbituric acid method.9

**Statistics**

Statistical analysis employed Student’s t test and determination of correlation coefficients.

**RESULTS**

**Phagocytosis**

Human marrow macrophages readily ingest sickle RBCs, while normal RBCs are relatively spared (Table 1). These data somewhat underestimate the degree of abnormality, since macrophages contain up to seven sickle RBCs per macrophage, while no more than one normal RBC is ingested per macrophage. The degree of sickle erythrophagocytosis actually exceeds that of RBCs from Coombs-positive control patients (Table 1).

Excessive phagocytosis of sickle RBCs is not explained simply by reticulocytosis, since RBCs from patients with posthemorrhagic reticulocytosis are ingested minimally (Table 1). Also, phagocytosis of sickle RBCs is strongly dependent upon RBC density, although even least-dense sickle RBCs are ingested more readily than normal RBCs (Table 2).

**K562 Rosetting**

Since acquisition of surface Ig is one mechanism that would promote abnormal RBC/macrophage interactions,17 we examined sickle RBCs for interactions with Fc-receptor-bearing K562 cells.16 These rarely form rosettes with normal RBCs but do so readily with Coombs-positive RBCs (Table 1). A small but significantly abnormal number of rosettes form with sickle RBCs, and none are seen for high-reticulocyte control RBCs (Table 1). Rosette formation is greatest for the most-dense (ISC-enriched) sickle RBCs (Table 2). Since the degree of RBC-K562 rosette formation is generally proportional to the amount of RBC IgG,16 these data indirectly demonstrate that sickle RBCs have abnormal amounts of surface Ig—but much less than Coombs-positive RBCs.

Blockade of K562 Fc receptors induced by human IgG (1 mg/mL) inhibits rosette formation with sickle RBCs by 97.2 ± 4.7% (n = 6) and that with Coombs-positive control RBC by 93.7 ± 9.1% (n = 4) (not shown in tables).

It should be noted that the K562 cells used for experiments in Tables 1 and 2 were derived from different sources. Those used for experiments in Table 2 were obtained from a line established from a later passage of the parent line, and they were found to be less active K562–RBC rosette formers. Although dif-

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**Table 1.** Macrophage and K562 Cell Interaction With Erythrocytes*

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>Macrophages ingesting RBCs (%)</th>
<th>K562-RBC Rosettes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal RBCs</td>
<td>3.1 ± 2.1 (14)</td>
<td>0.5 ± 1.2 (5)</td>
</tr>
<tr>
<td>Sickle RBCs</td>
<td>16.9 ± 7.2 (15)</td>
<td>15.6 ± 10.6 (7)</td>
</tr>
<tr>
<td>Coombs-positive RBCs†</td>
<td>12.8 ± 4.8 (5)</td>
<td>54.2 ± 3.6 (4)</td>
</tr>
<tr>
<td>High-reticulocyte RBCs‡</td>
<td>3.7 ± 0.6 (3)</td>
<td>0 ± 0 (3)</td>
</tr>
</tbody>
</table>

*Results are given as the mean ± SD (n).
†Anemic patients with positive direct Coombs test for IgG.
‡Patients with reticulocyte counts ranging from 3% to 20% because of hemorrhage.

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**Table 2.** Macrophage and K562 Cell Interactions With Density-Separated RBCs*

<table>
<thead>
<tr>
<th>Density (%)</th>
<th>Normal RBCs</th>
<th>K562-RBC Rosettes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Least-dense RBCs</td>
<td>4.3 ± 0.6 (3)</td>
<td>9.0 ± 3.0 (3)</td>
</tr>
<tr>
<td>Middle-layer RBCs</td>
<td>4.3 ± 0.6 (3)</td>
<td>12.3 ± 1.5 (3)</td>
</tr>
<tr>
<td>Most-dense RBCs</td>
<td>8.3 ± 4.0 (3)</td>
<td>25.3 ± 9.0 (3)</td>
</tr>
<tr>
<td>K562-RBC Rosettes</td>
<td>0 ± 0 (3)</td>
<td>1.3 ± 2.3 (3)</td>
</tr>
<tr>
<td>Least-dense RBCs</td>
<td>0.3 ± 0.6 (3)</td>
<td>11.3 ± 4.9 (3)</td>
</tr>
</tbody>
</table>

*Results are given as the mean ± SD (n). For macrophage studies, RBCs were separated using dextran and a discontinuous density gradient. For K562 studies, RBCs were separated using Percoll and a continuous density gradient. The top, middle, and bottom 20% density subpopulations were selected for study.
Table 3. Erythrophagocytosis Inhibition*

|                        | Number | Number of Macrophages Ingesting RBCs (% | Inhibition† (|%
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Coombs-positive RBCs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without FcR blockade</td>
<td>4</td>
<td>13.5 ± 5.2†&lt; P &lt; .05</td>
<td>= 0</td>
</tr>
<tr>
<td>With 1 mg/mL IgG</td>
<td>4</td>
<td>5.0 ± 2.2†</td>
<td>60.9 ± 14.3</td>
</tr>
<tr>
<td>With 5 mg/mL IgG</td>
<td>3</td>
<td>4.5 ± 0.7†</td>
<td>66.6 ± 5.4</td>
</tr>
<tr>
<td>Sickle RBCs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without FcR blockade</td>
<td>5</td>
<td>19.2 ± 6.4†&lt; P &lt; .02</td>
<td>= 0</td>
</tr>
<tr>
<td>With 1 mg/mL IgG</td>
<td>5</td>
<td>9.4 ± 3.7†</td>
<td>51.9 ± 7.6</td>
</tr>
<tr>
<td>With 5 mg/mL IgG</td>
<td>3</td>
<td>8.5 ± 2.1†</td>
<td>57.8 ± 8.1</td>
</tr>
<tr>
<td>Normal control RBCs</td>
<td>4</td>
<td>4.5 ± 0.6†</td>
<td></td>
</tr>
</tbody>
</table>

*Phagocytosis of sickle RBCs and Coombs-positive RBCs with and without blockade of macrophage Fc receptors (FcR) using 1 mg/mL or 5 mg/mL human IgG. Results shown as the mean ± SD.
†When results are expressed in terms of percentage of inhibition, sickle RBCs consistently show less inhibition of erythrophagocytosis by FcR blockade than do Coombs-positive RBCs; however, the difference is not statistically significant. As described in Materials and Methods, 1 mg/mL IgG is sufficient to inhibit formation of EA-rosettes with human macrophages by 54.0 ± 8.1%, and 5 mg/mL inhibits it by 64.3 ± 6.5%.

The latter observation argues against—but does not disprove—a role for RBC glycoproteins and amino-phospholipids in sickle erythrophagocytosis.

**Malondialdehyde**

Since sickle RBCs spontaenously generate approximately twice-normal amounts of activated oxygen,¹¹ RBCs were examined for spontaneous formation of dialdehyde byproducts of lipid peroxidation (“malondialdehyde” or MDA). Over 20 hours incubation, sickle RBCs (n = 5) generate 7.3 ± 2.6 nmol MDA per milliliter of RBCs, compared with only 3.9 ± 1.8 nmol for normal RBCs (n = 10) (P < .02). Since dialdehydes and lower monoaldehydes are known to promote erythrophagocytosis,²⁰ we evaluated the effect of MDA on RBC—macrophage interactions.

Treatment of normal RBCs with reagent-MDA induces a dose-dependent increase in phagocytosis by macrophages (Table 4). This effect could conceivably reflect some change in the availability of IgG already on the RBC surface. This possibility, however, is diminished by the fact that the MDA effect is virtually identical even when these experiments are repeated using RBCs devoid of surface IgG, such as those derived from the least-dense subpopulation of normal RBCs¹⁰ or those first subjected to heat elution procedures¹⁰ (data not shown). That endogenous dialdehydes may have the same effect as reagent-MDA is supported by the fact that erythrophagocytosis is enhanced by exposure of normal RBCs to 10 mmol/L H₂O₂ and by prolonged incubation of sickle RBCs (Table 4).

Since reagent-MDA is a bifunctional aldehyde that crosslinks amino groups, two additional controls were used. Erythrophagocytosis is not diminished by prior incubation of MDA-treated RBCs with either 10 mmol/L lysine or 1% albumin, each of which would block any incompletely reacted MDA that might

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"Glucose, galactose, ribose, fructose, mannose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylneuraminic acid, serine, ethanolamine, choline; each at 25 mmol/L final concentration."
crosslink RBCs to the macrophage (data not shown). On the other hand, the phagocytosis-promoting effect of 1 mmol/L MDA is completely abolished by including 10 mmol/L lysine in the initial treatment mixture to compete with RBC membrane amino groups for reaction with MDA (data not shown).

Interestingly, other aldehydes (one bifunctional, one monofunctional) have no effect upon erythrophagocytosis, even at 1 mmol/L concentration (Table 4). Similarly, equimolar amounts of a nonaldehyde crosslinker (carbodiimide) are also without effect (Table 4). These experiments thus suggest that there may be something unique about membrane modification by MDA.

### Possible Convergence of Mechanisms

Reagent-MDA also appears to promote erythrophagocytosis by a second mechanism. Above and beyond the above-described Ig-independent effect of MDA treatment alone, subsequent exposure of MDA-treated RBCs to human serum containing Ig results in a significant additional increase in erythrophagocytosis (Table 5). This latter effect is diminished by Fc receptor blockade (Table 5) and indicates that MDA-induced membrane modification increases amounts of IgG on the RBC surface. If this were due to MDA modification exposing a "cryptic antigen" (or itself being antigenic), incubation of MDA-treated RBCs with human IgG Fab fragments before serum exposure might prevent the subsequent attachment of IgG and therefore prevent enhancement of erythrophagocytosis by serum. Indeed, prior exposure to Fab fragments (10 mg/mL) does inhibit the phagocytosis-enhancing effect of serum upon MDA-treated RBCs (Table 5).

### Adherence Studies

The erythrophagocytosis assay was found to be too crude for comparison of different sickle patients. For example, even though sickle RBCs are always ingested more readily than normal RBCs, preliminary experiments revealed that the sickle patients whose RBCs are ingested to the greatest extent after short incubation (eg, three hours) are not necessarily the patients

### Table 4. Erythrophagocytosis of Manipulated Erythrocytes

<table>
<thead>
<tr>
<th>Manipulated Normal RBCs: Malondialdehyde (MDA)</th>
<th>Macrophages Ingesting RBCs (%)</th>
<th>Percent</th>
<th>Number</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.6 ± 0.5</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA, 20 μmol/L</td>
<td>6.0 ± 1.7</td>
<td>3</td>
<td>&lt;.1</td>
<td></td>
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<tr>
<td>MDA, 100 μmol/L</td>
<td>7.3 ± 0.6</td>
<td>3</td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>MDA, 500 μmol/L</td>
<td>8.0 ± 2.0</td>
<td>3</td>
<td>&lt;.05</td>
<td></td>
</tr>
<tr>
<td>MDA, 1 mmol/L</td>
<td>9.6 ± 1.5</td>
<td>3</td>
<td>&lt;.001</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Manipulated Normal RBCs: Other aldehydes/crosslinkers</th>
<th>Macrophages Ingesting RBCs (%)</th>
<th>Percent</th>
<th>Number</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.3 ± 1.5</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde, 1 mmol/L</td>
<td>6.0 ± 1.0</td>
<td>3</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde, 1 mmol/L</td>
<td>6.3 ± 1.2</td>
<td>3</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Carbodiimide, 1 mmol/L</td>
<td>4.7 ± 0.6</td>
<td>3</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Manipulated Normal RBCs: Peroxide treatment</th>
<th>Macrophages Ingesting RBCs (%)</th>
<th>Percent</th>
<th>Number</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.3 ± 1.5</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O₂, 10 mmol/L</td>
<td>11.0 ± 0</td>
<td>3</td>
<td>&lt;.01</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>In vitro &quot;aging&quot;</th>
<th>Macrophages Ingesting RBCs (%)</th>
<th>Percent</th>
<th>Number</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal RBCs, Control</td>
<td>2.0 ± 1.4</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal RBCs, Aged</td>
<td>5.0 ± 1.4</td>
<td>3</td>
<td>&lt;.1</td>
<td></td>
</tr>
<tr>
<td>Sickle RBCs, Control</td>
<td>13.5 ± 0.7</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sickle RBCs, Aged</td>
<td>24.0 ± 8.5</td>
<td>3</td>
<td>&lt;.1</td>
<td></td>
</tr>
</tbody>
</table>

*Results are given as mean ± SD with P value compared with own control (NS, not significant).

### Table 5. Possible Convergence of Oxidative and Immunologic Mechanisms Promoting Erythrophagocytosis

<table>
<thead>
<tr>
<th>Manipulated Normal RBCs</th>
<th>Macrophages Ingesting RBCs (%)</th>
<th>Percent</th>
<th>Number</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control incubations</td>
<td>3.8 ± 0.7 (9)</td>
<td>P &lt; .001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mmol/L MDA-treated</td>
<td>9.0 ± 1.0 (9)</td>
<td>P &lt; .02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>followed by serum exposure</td>
<td>12.6 ± 3.4 (8)</td>
<td>P &lt; .01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 1 mmol/L MDA-treated</td>
<td>followed by serum exposure but assayed with FcR blockade (1 mg/mL IgG)</td>
<td>6.3 ± 1.2 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. 1 mmol/L MDA-treated</td>
<td>followed by serum exposure but incubated with Fab fragments before serum exposure</td>
<td>6.1 ± 0.8 (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Results are given as mean ± SD (n) with P values. The phagocytosis-promoting effect of serum upon MDA-treated RBCs (line 3 v line 2) is blocked by two manipulations: (1) induction of Fc receptor [FcR] blockade during erythrophagocytosis assay (line 4); and (2) exposure of MDA-treated RBCs to Fab fragments of normal human IgG before serum exposure (line 5).
whose RBCs are most extensively ingested after longer incubation (eg, 15 hours). Although erythropagocytosis undoubtedly involves a number of critical steps, we reasoned that propensity for initial contact between RBCs and macrophages would necessarily be the first rate-limiting step. Consequently, we utilized the short adherence assay for comparison of different patients.

Erythrocytes from 14 sickle cell anemia patients are 2.93 ± 1.78 times as adherent as normal RBCs to marrow macrophages, with “macrophage adherence ratios” ranging from 1.03 to 6.85. Among these sickle patients, propensity for macrophage adherence correlates significantly with percentage of ISC and with hematologic variables reflecting hemolytic rate (Table 6). Fc receptor blockade with 1 mg/mL IgG inhibits sickle RBC adherence to macrophages by 81.6 ± 31.9% (n = 3), and MDA treatment of normal RBCs enhances their adherence by 1.7 ± 0.3-fold (n = 3). Adherence is also density-dependent, with most-dense sickle RBCs being 2.4 ± 1.3-fold (n = 3) more adherent to macrophages than least-dense sickle RBCs.

For RBCs from these 14 sickle patients, there is no correlation between macrophage adherence and endothelial adherence (r = −.101). Unlike RBC adherence to macrophages, adherence of sickle RBCs to endothelium is not diminished by Fc receptor blockade and does not correlate with ISC count, reticulocyte count, or hemoglobin concentration (data not shown).

Nonmarrow Macrophages

Several experiments demonstrated that sickle RBCs are also abnormally adherent to—and phagocytosed by—alveolar and splenic macrophages. In addition, comparison of three sickle patients with low, moderate, and high degrees of adherence to marrow macrophages revealed the same relative relationships for adherence to alveolar macrophages (data not shown). Thus, there is nothing unique about marrow macrophages in this regard.

DISCUSSION

Interpretation of Results

The reason for accelerated RBC destruction in sickle disease is incompletely understood. Irreversibly sickled cells (ISC) are shorter-lived than non-ISCs, and hemolytic rate is reported to be proportional to ISC count. However, the precise mechanism by which ISC formation is accompanied by premature removal of RBCs is uncertain. Recent data have suggested that factors in addition to (or instead of) poor deformability or abnormal shape may underlie splenic removal of RBCs, and RBC survival in sickle patients remains greatly attenuated despite childhood “autosplenectomy.” As discussed in the introduction, the major portion of sickle hemolysis may be attributable to participation of the reticuloendothelial system.

The present data indicate that sickle RBCs have an abnormal propensity for adherence to and phagocytosis by macrophages and may help explain accelerated RBC destruction in sickle disease. Although most experiments employed marrow macrophages because of their greater availability, additional studies demonstrated that sickle RBCs also interact in similar fashion with alveolar and splenic macrophages. Thus, the excessive phagocytosis of sickle RBCs may reflect a universal characteristic of macrophages rather than one of restricted tissue specificity. These studies were not intended to specifically determine whether the same RBC abnormality predisposes toward adherence
and phagocytosis. The data are consistent with this possibility, however, since adherence and phagocytosis were found to have the same characteristics: both increase with sickle RBC density (although least-dense RBCs are also abnormal); both are inhibited by Fc receptor blockade; and both can be induced by reagent-MDA treatment of normal RBCs.

These findings suggest two possible mechanisms for the abnormal interaction between sickle RBCs and macrophages. First, its partial inhibition by Fc receptor blockade suggests it is in part an immunologic event, initiated by macrophage recognition of Ig on the RBC surface. This is supported by the observation of excessive rosette formation between sickle RBCs and (Fc-receptor-bearing) K562 cells. Notably, using quite different techniques Petz et al26 have preliminarily reported that sickle RBCs have abnormal amounts of surface IgG.

An additional mechanism underlying this abnormal cell–cell interaction may be excessive sickle RBC autoxidation. As shown here, sickle RBCs spontaneously generate approximately twice-normal amounts of dialdehyde byproducts of lipid peroxidation ("MDA"), presumably because of their relentless generation of activated oxygen,11 and evidence for cross-linking of membrane amino groups by "MDA" has been found in sickle RBC membranes.27,28 We find that treatment of normal RBCs with even micromolar concentrations of reagent-MDA promotes their phagocytosis. Laboratory exposure of RBCs to other dialdehydes and lower monoaldehydes (at much higher concentrations) has previously been shown to enhance erythrophagocytosis.20 We find that reagent-MDA is at least 50 times more potent in this regard. In addition, phagocytosis is enhanced by exposure of normal RBCs to nonlytic amounts of peroxide and by prolonged incubation of sickle RBCs (both of which promote formation of endogenous dialdehyde byproducts of lipid peroxidation). Thus, physiologic dialdehydes to which cell membranes are exposed during autoxidation may promote phagocytosis.

This opsonic effect of MDA was evident even if the experiments utilized RBCs known to be devoid of IgG, such as those from the least-dense subpopulation of normal RBCs10 or those previously subjected to heat elution of Ig.10 Hence, one mechanism by which macrophages recognize MDA-modified RBC membranes appears to be Ig-independent. The precise nature of this mechanism remains obscure, but it has been shown that macrophages recognize MDA-modified lipoproteins through their so-called "scavenger receptor."29 An alternative (and not incompatible) mechanism might involve enhanced hydrophobicity of the RBC membrane induced by aldehydes.20

Of particular interest, our data also suggest convergence of immunologic and oxidative mechanisms, as evidenced by an additional Ig-dependent effect of MDA. Phagocytosis of MDA-treated normal RBCs is further enhanced if they are exposed to Ig-containing sera, an effect that is inhibited by Fc receptor blockade. This suggests that modification of the RBC membrane by MDA enhances IgG binding. That this represents a true immunologic attachment (rather than passive, nonimmunologic adsorption) of IgG is suggested by the fact that prior incubation of MDA-treated RBCs with Fab fragments from human IgG prevents the phagocytosis-promoting effect of serum. Our data do not distinguish between this being the result of exposure of a pre-existing "cryptic" antigen versus formation of a truly new MDA-specific antigen. It also will have been noted that this prior incubation with Fab fragments reduced phagocytosis of MDA-treated/serum-exposed RBCs down to levels even lower than those of MDA-treated RBCs. This too is consistent with convergence of mechanisms, in that it may indicate that Fab fragment attachment also masks the sites involved in Ig-independent promotion of phagocytosis by MDA. The slight diminution of sickle erythrophagocytosis by Fab fragments (at tenfold lower concentration) may reflect this same process.

That this interaction between sickle RBCs and macrophages may contribute to the hemolytic component of sickle disease is suggested by the significant relationship between propensity for RBC adherence to macrophages and hematologic variables reflecting hemolytic rate. In addition, sickle RBC adherence to macrophages correlates significantly with ISC count, which is consistent with existing data implicating ISC in hemolytic rate.21-23 The ISC-enriched (most-dense) fraction of sickle RBCs is here shown to be most adherent to—and most readily ingested by—macrophages. Yet, ISC-depleted fractions still manifest these abnormal interactions.

Precisely which mechanisms are actually involved in RBC–macrophage interactions in vivo cannot, of course, be inferred from these studies. Indeed, additional membrane abnormalities of sickle RBCs might be involved. For example, other investigators have preliminarily suggested that abnormalities of the lipid bilayer may promote attachment of sickle RBCs to monocytes.30 Of interest, even though most-dense sickle RBCs manifest the greatest adherence to both endothelial cells31 and macrophages, different membrane lesions appear to predispose toward these two abnormal cell–cell interactions. For example, adherence to macrophages correlates with variables reflecting hemolytic rate, while adherence to endothelium
does not\(^8\); and macrophage adherence is inhibited by Fc receptor blockade, while endothelial adherence is not.

**Speculation**

Regarding the broader biologic significance of these data, we are intrigued by the possibility that sickle cell disease is, to some extent, an accelerated form of normal processes of RBC membrane senescence.\(^11\) If this is true, macrophages may recognize sickle RBCs not simply as being abnormal, but as being “senescent.” In this regard, Kay’s elegant studies\(^8,9,32\) have suggested that normal RBC senescence involves attachment of Ig directed at a newly-exposed “senescence antigen,” which is responsible for macrophage recognition of old RBCs and results in their removal from the circulation. Kay has further suggested that “senescence antigen” formation may involve some modification of Band III protein.\(^32\) Insofar as autooxidation might be involved in aging of normal RBCs, we are tempted to suggest that this modification of Band III involves the dialdehyde byproducts of lipid peroxidation (“MDA”). Indeed, the present data are consistent with this hypothesis in that subsequent exposure of MDA-treated normal RBCs to Ig-containing sera further enhances erythropagocytosis, an effect that is inhibited by Fc receptor blockade and that is prevented by Fab fragments of IgG found in normal sera.

Finally, these data perhaps add a new dimension to our concept of autoimmune hemolytic anemia, which is classically considered to reflect appearance of a new antibody to an existing antigen. Our data suggest that accelerated removal of pathologic RBCs may, in a certain sense, be an “autoimmune” event to the extent that an existing antibody (perhaps the physiologic autoantibody studied by Kay\(^8,9,32\)) might become problematic upon appearance of a new antigen (ie, physiologic alteration of the RBC membrane). Whether the IgG indirectly demonstrated on sickle RBCs by these studies is, in fact, identical to the physiologic Ig found on senescent normal RBCs remains to be shown. Nevertheless, these findings regarding sickle RBCs may be relevant to mechanisms of hemolysis in other disorders classically described as nonimmunologic. Examples might be patients with thalassemia and malaria, RBCs from whom also interact abnormally with macrophages in vitro.\(^6,7\)

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Phagocytosis of sickle erythrocytes: immunologic and oxidative determinants of hemolytic anemia

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