In this study we attempt to establish the consequence of in vitro hydrogen peroxide (H$_2$O$_2$)-induced membrane damage as manifested by spectrin-hemoglobin (Sp-Hb) complex formation and decreased red blood cell (RBC) deformability to in vivo RBC survival in baboons. After exposure to 135 to 581 µmol/L H$_2$O$_2$ and reduction with dithiothreitol (DTE), baboon RBCs were infused into the animal, and the fraction of cells remaining in circulation after 24 hours and the lifespan of surviving cells were quantitated. In a dose-dependent fashion, a positive correlation was observed between in vitro membrane alterations and the 24-hour in vivo survival. While 12% of the control cells were removed from circulation in 24 hours, 23% were removed after treatment with 339 µmol/L H$_2$O$_2$, and 36% following exposure to 581 µmol/L H$_2$O$_2$. Pretreatment with carbon monoxide before exposure with H$_2$O$_2$ increased the survival of oxidized RBCs. RBCs not removed from circulation in the first 24 hours had a normal lifespan. Moreover, by selectively isolating biotin-labeled, peroxide-treated cells from circulation, the hypothesis that oxidative damage to the RBC membrane may play an important role in cell senescence has attracted much attention. For example, it has been suggested that during early stages of aging, hemoglobin (Hb) becomes associated with the membrane, and that subsequent oxidation of this membrane-associated hemoglobin results in the generation of free radicals, which in turn initiate irreversible membrane alterations. Support for this suggestion are recent studies of RBC aging in the hypertransfused mouse model, in which a consistent marker of cell senescence was found to be the retention of globin by the RBC membrane skeleton. In addition, we have identified a crosslinked complex between spectrin (Sp) and globin in the most dense human RBCs isolated from whole blood, and have shown that the generation of this protein complex results in decreased RBC deformability. Exposure of human RBCs to low concentrations of hydrogen peroxide (H$_2$O$_2$) in vitro results in similar alterations, including the generation of Sp-Hb complex, decreased RBC deformability, and cell surface alterations, which lead to enhanced phagocytosis of the oxidized cells by monocytes. Importantly, prior treatment of human RBCs with carbon monoxide completely inhibited cellular alterations induced by H$_2$O$_2$. In this study we attempt to determine the in vivo consequences of this form of oxidatively induced damage to RBCs by using an animal model. Because cellular and survival characteristics of baboon RBCs are similar to those of human RBCs, we chose to perform the in vivo studies in the baboon. After establishing that in vitro peroxidative damage induced in baboon RBCs is similar to that in human RBCs, we quantitated the in vivo survival of oxidatively damaged baboon RBCs. We found that the decrease in 24-hour posttransfusion survival of damaged RBCs correlated positively with the extent of Sp-Hb complex generated, alterations in cell deformability, and hence the extent of oxidative damage.

**MATERIALS AND METHODS**

Preparation of oxidatively damaged RBCs. Samples of venous blood were obtained from baboons using citrate-phosphate-dextrose (CPD) as the anticoagulant. After centrifugation to remove...
plasma and leukocytes, the RBCs were washed once with phosphate-buffered saline (PBS: 5 mmol/L phosphate, 0.15 mol/L NaCl, and 5 mmol/L dextrose, pH 7.4) and resuspended to a final hematocrit of 20% in PBS with 2 mmol/L sodium azide to inhibit catalase. After incubation at 30°C for 10 minutes, measured quantities of H₂O₂ were added to the RBC suspension to obtain final concentration in the range of 135 to 581 μmol/L, and the cells were incubated for an additional 10 minutes at 30°C. The oxidative reaction was quenched by washing the cells with PBS containing 5 mmol/L dithiothreitol (DTE), followed by a 5-minute incubation in the same buffer. Control RBCs underwent the same regimen with one exception: no H₂O₂ was added to these cells. Controls were also run in the absence of DTE.

In some experiments RBCs were pretreated with carbon monoxide before oxidation with H₂O₂. Eight milliliters of 20% hematocrit RBC suspension was exposed to carbon monoxide (500 mL/min) in a tonometer (Model 227; Instrumentation Labs, Watertown, MA) for 15 minutes. When carboxy-Hb levels were greater than 95%, H₂O₂ was added to the reaction vessel to initiate oxidant damage. The treatment of carbon monoxide-treated cells with H₂O₂ and subsequent processing was identical to that outlined above for normal RBCs.

To demonstrate that in vitro peroxidation had no effect on the rate of subsequent in vitro Cr 51 elution from labeled RBCs, CR51-labeled control and peroxide-treated cells were incubated for 24 hours in autologous plasma, and the Cr 51 counts released into the supernatant were measured.

In vivo RBC survival determination. Experimental manipulations involving RBCs used for in vivo lifespan measurement were performed under sterile conditions. The various solutions used during the procedure were sterilized either by steam autoclaving or by filtering through a 0.2-μm/L filter (MSI, Westborro, MA). Penicillin G (2,500 U/mL) was added to the PBS buffer used for washing RBCs before reinfusion. The sterility of the treated RBC suspension was determined by microbiologic testing using blood agar plates to detect aerobic organisms, and thioglycollate broth tubes for detecting both aerobic and anaerobic organisms. RBC survival was quantitated using the double CR51 labeling method previously described. Twenty-four-hour postinfusion survival (%) and RBC lifespan (½) were calculated by monitoring the loss of CR51-labeled RBCs from circulation. Means and ± 1 SD were calculated for each experimental group, and comparisons between groups were made using the Student's t-test. Due to the limited number of animals, some baboons were reused after a reasonable period of time (3 to 4 months) for subsequent oxidation survival studies.

Electrophoretic analysis of membrane proteins. The membrane ghosts were prepared by hypotonic hemolysis as described previously. Equal amounts of sodium dodecyl sulfate (SDS)-saturated membrane protein obtained from a defined number of ghosts were run on cylindrical 4% polyacrylamide gels described previously. The percentage of Sp-Hb complex was calculated by integrating the Coomassie blue stain profile on the densitometric scans from H₂O₂-treated RBC ghosts in comparison with scans obtained from control cells, and was expressed as a percentage of total Sp.

Membrane deformability measurements. For deformability measurements, resealed membranes from untreated and oxidized RBCs were prepared by a procedure adopted from Mohandas et al. RBCs were washed three times in 5 ml/L Tris, 140 mm NaCl (pH 7.4), and then lysed in 40 vol of 7 mmol/L NaCl and 5 mmol/L Tris (pH 7.4). The membranes then were pelleted by centrifugation, resuspended in 10 vol of 5 mmol/L Tris and 140 mmol/L NaCl (pH 7.4), and incubated for 30 minutes at 37°C for resealing.

For deformability measurements, resealed membranes, prepared as described above, were suspended in 3 ml of Stractan II (290 mOsm, 22 cp, pH 7.4; Sigma Chemical Co, St Louis, MO) and exposed to an increasing shear stress (0 to 125 dynes/cm²) in the ektacytometer. By an automatic image analysis system, the deformability index (DI) is recorded as a continuous function of applied shear stress. Analysis of the DI curve generated by the ektacytometer provides a measure of the dynamic deformability of the RBC membrane.

Separation of RBCs on density gradients. RBCs of defined density characteristics were isolated using Percoll-Hypaque density gradients. Forty-five milliliters of a packed suspension of washed baboon RBCs was mixed thoroughly with a mixture of Percoll and Hypaque (325 mL Percoll, 325 mL distilled water, 160 mL Hypaque) and spun for 20 minutes at 15,000 rpm in a centrifuge (Sorvall, ss-24 rotor; DuPont Sorvall, Wilmington, DE). After spinning, the uppermost layer of cells on the gradient, consisting predominantly of white blood cells and reticulocytes, was aspirated and discarded. The top 10% and bottom 10% of the RBCs in the gradient were carefully isolated, washed three times in PBS, and used for further analysis.

Preparation and recovery of surviving, peroxide-treated, biotinylated baboon erythrocytes at 24 hours posttransfusion. Biotinylated baboon RBCs were prepared by a procedure adopted from Dale and Norenberg. All reagents used in this procedure were obtained from Sigma Chemical Co. One hundred twenty milliliters of blood was obtained from a male baboon. The RBCs were treated with H₂O₂ (581 μmol/L), washed three times in PBS, and were resuspended to a 10% hematocrit in PBS containing 15 mmol/L glucose. The RBCs were then incubated at 37°C for 15 minutes. Succinylated bovine serum albumin (BSA), 295 nmol, was added to the cell suspension, mixed thoroughly, and incubated for 3 minutes at 37°C. This was followed by the addition of NHS-biotin (295 nmol) and incubated at 37°C for an additional 60 minutes. The biotinylated RBCs were washed three times in PBS, Cr51-labeled, and reinfused into the baboon. At 24 hours posttransfusion, 60 mL of whole blood was drawn and washed several times in PBS. The RBCs were resuspended to a 35% hematocrit in PBS containing 1 mg/mL BSA and 5 mmol/L glucose.

The surviving biotinylated RBCs were recovered on avidin plates (15-cm polystyrene Petri dishes, cat. no. d1905; Scientific Products, McGraw Park, IL). The avidin plates were prepared by adding 1.8 mg of biotinylated gelatin in 5 mL of PBS to each plate. The plates were then gently rocked overnight. Forty milligrams of BSA was then added and the plates were rocked for 30 minutes. The plates were then washed several times with PBS, and 0.4 mg of avidin in 5 mL of PBS was added to each plate and placed on the rocker for 60 minutes. The avidin plates were then washed clean with PBS. Three milliliters of the cell suspension was added to each plate and gently rocked for 60 minutes. Unbound RBCs were gently rinsed from the dish with a transfer pipette and PBS. The bound RBCs were removed from each plate by adding 5 mL of PBS containing 0.2 mg/mL collagenase (type IV, 445 U/mL), 0.15 mg/mL BSA, and 0.1 mmol/L biotin and by placing on the rocker for 15 minutes. Some RBCs required gentle agitation with a transfer pipette to release from the plate. The recovered RBCs were then washed several times, and 51 counts/g of hemoglobin were determined, and the cells were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Some plates were treated with 5 mL of 10 mmol/L biotin before the addition of biotinylated RBCs to measure nonspecific binding. To evaluate the efficacy of H₂O₂-treated biotinylated RBCs to bind to avidin plates, some cells were washed after biotinylation and diluted 1:15 to account for the blood volume dilution in the baboon, and were placed directly onto the avidin plates. The bound RBCs were washed in PBS and prepared for SDS-PAGE.

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RESULTS

The results of in vitro measurements to assess the oxidative damage in the RBC membrane, such as the formation of spectrin-hemoglobin complex and changes in RBC deformability, showed that the baboon and human RBCs were slightly different in their respective responses to oxidative stress.

Table 1 and Fig 1 show the effect of H2O2 on the formation of Sp-Hb crosslinking and changes in deformability. In a dose-dependent fashion starting at 242 μmol/L, an increase in Sp-Hb crosslinking was noted in baboon RBCs. Sp-Hb crosslinking was detected as an additional diffuse band migrating at the trailing edge of the α chain of Sp with loss of the acute angle between the trailing edge of band 1 and the baseline on densitometric scans, with a molecular weight of 255 Kd. The increase in Sp-Hb crosslinking was also associated with an increase in RBC membrane rigidity as measured by ektacytometry. However, for the same degree of Sp-Hb crosslinking, the baboon RBC was less sensitive than the human RBC to changes in deformability.

Table 2 demonstrates the amount of Sp-Hb complex formation in Percoll-Hypaque gradient separated baboon RBCs with or without treatment with 339 μmol/L H2O2. As seen in human RBCs, an incremental increase in Sp-Hb complex formation from the least dense to the most dense RBCs was also observed in baboon RBCs. Complex formation was also positively correlated with an increase in RBC membrane rigidity. After treatment with 339 μmol/L H2O2, a more marked increase in complex formation and increased membrane rigidity was seen for the dense bottom cells. Because the above data clearly established the similarities as well as the differences between the human and baboon RBC in their response to oxidative damage, we then proceeded to test the effects of in vitro oxidative perturbation on the in vivo survival of baboon RBCs.

Twenty-four-hour in vitro elution studies demonstrated that there was essentially no significant difference in the number of Cr51 plasma counts between peroxide-treated (4.6% ± 0.7% of total counts, n = 3) and control (5.5% ± 1.7%, n = 3) cells, indicating that oxidation does not result in differential elution of Cr51 from oxidized baboon RBCs.

Table 3 demonstrates the in vivo survival of H2O2-treated baboon RBCs. The 24-hour posttransfusion data demonstrated a progressive and significant decrease in survival with increasing H2O2 concentrations; comparative P values ranged from .01 to .05, and correlated well with the in vitro observation of H2O2-induced increase in Sp-Hb crosslinking and changes in membrane rigidity. The Cr51 t1/2 of the RBCs in the circulation 24 hours after transfusion was normal in all samples. Pretreatment with carbon monoxide before oxidation with 484 μmol/L H2O2 increased the 24-hour posttransfusion survival from a mean of 70.7% ± 1.7% in oxidized cells to 81.5% after carbon monoxide exposure; this difference was significant at the P < .05 level. Control cells run with and without DTE showed no significant differences with regard to 24-hour posttransfusion survival. Re-exposure of baboons to additional H2O2-RBCs did not result in alterations in either the predicted 24-hour posttransfusion survival or Cr51 t1/2.

Baboon RBCs incubated with 581 μmol/L H2O2, labeled with Cr51, and reinjected resulted in a slight decrease in Cr51 survival during the 30-minute posttransfusion period, with a significant difference after 24 hours between control and oxidized samples. A representative study is illustrated in Fig 2.

Table 4 demonstrates that unfractionated RBCs exposed to Percoll-Hypaque (control) had normal 24-hour posttransfusion survivals as well as Cr51 t1/2 values. The untreated top 10% (MCHC < 32 g/dL) and the untreated bottom 10% (MCHC > 37 g/dL) RBCs showed no difference in either 24-hour posttransfusion survival or Cr51 t1/2 values. However, after treatment with 339 μmol/L H2O2, the 24-hour posttransfusion survival of bottom cells was significantly decreased compared with treated and untreated top cells (P < .05), and this decrease in survival was paralleled by increased Sp-Hb complex formation and increased membrane rigidity. The survival of treated bottom cells was...
Table 3. In Vivo Survival of H2O2 Treated Baboon Erythrocyte

<table>
<thead>
<tr>
<th>Survival</th>
<th>Control</th>
<th>DTE</th>
<th>H2O2 μmol/L</th>
<th>CO + H2O2 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-h posttransfusion</td>
<td>88.7 ± 4.1 n = 3</td>
<td>88.0 ± 1.0 n = 3</td>
<td>76.75 ± 1.7 n = 4</td>
<td>70.75 ± 1.7 n = 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>81.82 n = 2</td>
<td>64.5 ± 5.9 n = 4</td>
</tr>
<tr>
<td>Cr51 t/2 (d)</td>
<td>13.0 ± 1.1 n = 3</td>
<td>15.0</td>
<td>15.1, 14.7 n = 3</td>
<td>14.1, 12.9</td>
</tr>
<tr>
<td>Sp-Hb complex (% of total Sp)</td>
<td>0</td>
<td>3.7, 5.5</td>
<td>6.0</td>
<td></td>
</tr>
</tbody>
</table>

In the current study we show the similar effects of oxidation on baboon and human RBCs treated with H2O2. Incubation of baboon RBCs with H2O2 resulted in dose-dependent Sp-Hb complex formation associated with increased membrane rigidity similar to that seen in human RBCs. As in human RBCs, pretreatment of baboon RBCs with carbon monoxide prevented the complex formation as well as alterations in membrane deformability. In the present study we attempted to establish the consequence of the in vitro-induced H2O2 membrane damage by studying the in vivo survival of the damaged cells. The decrease in survival of 24-hour posttransfusion baboon erythrocytes incubated with 339 μmol/L to 581 μmol/L concentrations of H2O2 provided in vivo evidence that 24-hour posttransfusion survival was related to peroxide-induced membrane damage.

Pretreatment of cells with carbon monoxide protects the in vivo survival of peroxide-treated cells, and substantiates our previous postulation that Hb oxidation is an important step in subsequent membrane alterations. The H2O2-induced membrane alterations were not reversed by the reducing agent dithiothreitol; this observation was similar to observations with human erythrocytes in vitro and it is in contrast to the reversible membrane damage induced by diamide observed in human and dog RBCs in vivo.

Reusing baboons for repeat oxidation experiments with varying concentrations of H2O2 does not alter either the Cr51 t/2 or the predicted 24-hour posttransfusion survival data.
These results support the hypothesis that the membrane lesions associated with peroxidation are more focal lesions involving the membrane skeletal network of proteins rather than altering the RBC surface in such a way as to create new antigenic sites, which results in the cells rapid removal by an immunologic mechanism.

The relationship between the formation of Sp-Hb crosslinking and shortening of the 24-hour posttransfusion survival of H$_2$O$_2$-treated baboon RBCs is further supported by studies in which surviving biotinylated H$_2$O$_2$-treated RBCs were selectively isolated. The surviving 24-hour posttransfusion H$_2$O$_2$-treated RBCs have normal Cr51 t½ and show a significant decrease in Sp-Hb crosslinking. This demonstrates further that the cells removed during the first 24 hours are the cells that carry primarily the membrane skeletal protein damage.

This data suggests that there is a subpopulation of oxidant-sensitive erythrocytes that is removed during the first 24 hours. The remaining oxidant-resistant cells have a normal Cr51 t½. This could be explained by the fact that (1) the damage to the remaining cells was minimal, and/or (2) that a reparative process was able to remove the damaged membrane and thus allow the remaining RBCs to survive normally.

In an attempt to define this population of sensitive cells, we studied oxidized and control baboon RBCs of different densities. The fact that we were unable to demonstrate a difference in survival between the untreated top 10% (MCHC ≤ 32 g/dL) and the bottom 10% (MCHC ≥ 37 g/dL) is interesting, and supports the recent finding in the mouse system in which little increase in cell density was noted during the normal RBC aging. This finding further challenges the dogma that RBC density and cell age are directly related. However, we did demonstrate that there was a difference between the oxidant sensitivity of the top and bottom cells. The bottom 10% of the cells were more sensitive to oxidative damage as manifested by in vitro alterations in membrane function as well as a substantial decrease in in vivo survival.

The question of whether decreased RBC deformability leads to hemolysis is still unanswered. However, if a significant contribution to the alteration in deformability is due to an increase in membrane rigidity as a result of globin complexing to membrane skeletal proteins, then the alteration in deformability as seen in H$_2$O$_2$-treated RBCs may play a role in removal of these cells.

Thus, the observations made in this study support our hypothesis that oxidant-induced erythrocyte membrane injury as manifested by the formation of Sp-Hb complex associated with decreased deformability is pathophysiologically significant, and results in in vivo hemolysis, particularly in the more dehydrated cells. However, this hypothesis does not preclude additional membrane proteins from being affected by oxidant damage. Therefore, the formation of Sp-Hb crosslinking may represent a more widespread oxidant damage. Moreover, the baboon is an excellent model system in which to test various in vitro hypothesis of RBC senescence.

REFERENCES


Table 4. In Vivo Survival of Percoll-Hypaque Density Separated Baboon Erythrocytes Treated With and Without 339 pmol/L H$_2$O$_2$

<table>
<thead>
<tr>
<th>Survival Values</th>
<th>Control</th>
<th>Top 10%*</th>
<th>Top 10%†</th>
<th>Bottom 10%</th>
<th>Bottom + H$_2$O$_2$</th>
<th>Bottom + H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-h posttransfusion</td>
<td>86.8 ± 6.4</td>
<td>86.85</td>
<td>89.0 ± 5.7</td>
<td>82.4 ± 6.3</td>
<td>70.0 ± 6.7</td>
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</tr>
<tr>
<td>Cr51 t½ (d)</td>
<td>12.4 ± 1.4</td>
<td>14.8, 14.6</td>
<td>14.0 ± 2.8</td>
<td>14.4 ± 2.0</td>
<td>12.8, 14.3</td>
<td></td>
</tr>
<tr>
<td>Sp-Hb complex (%)</td>
<td>0</td>
<td>0</td>
<td>3.9 ± 0.95</td>
<td>1</td>
<td>9.5</td>
<td></td>
</tr>
</tbody>
</table>

* MCHC 32 g/dL; 32 ± 0.3, n = 3. † MCHC 37 g/dL; 37 ± 1.03, n = 4.


Decreased in vivo survival of hydrogen peroxide-damaged baboon red blood cells

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