Hybrid Erythrocytes for Membrane Studies in Sickle Cell Disease

By Margaret R. Clark and Stephen B. Shohet

A hybrid erythrocyte model for membrane studies in sickle cell disease has been developed. The model consists of normal red cell membranes containing hemoglobin S and sickle cell membranes containing hemoglobin A. In hybrids, complete hemoglobin exchange has been achieved together with restoration of low membrane permeability to potassium. Normal membranes containing HbS sickle upon deoxygenation and assume the characteristic appearance of irreversibly sickled cells (ISC) after prolonged anoxia. It is suggested that the hybrid model will be useful in defining further the process of ISC formation and in studying the influence of sickle hemoglobin upon the function of the surrounding membrane.

Although sickle cell disease is well understood as an abnormality of the hemoglobin molecule, the complete mechanism by which this defect is expressed at the clinical level is not fully understood. A considerable body of evidence suggests that defective function of the sickle cell membrane may contribute to some of the manifestations of the disease. The membrane defect is visibly demonstrated in the permanently deformed morphology of the irreversibly sickle cell (ISC) and is also expressed as an increased cation leak from sickle cells when they are deoxygenated. Although partially characterized, the genesis of the membrane defect remains unknown. Various suggested mechanisms have emphasized the importance of repeated sickling, prolonged contact of the membrane with sickle hemoglobin, and abnormal concentrations of divalent cations or glycolytic intermediates. However, the roles of these factors have not yet been defined.

In an effort to characterize further the membrane defect and to elucidate the mechanisms by which it develops, we have devised a system for observing the effect of sickling upon membranes which have never before sickled. In addition, the model allows introduction of substances which may modify the process of membrane damage. The model consists of “hybrid” erythrocytes, composed of membranes from normal cells surrounding hemoglobin S and sickle cell membranes surrounding hemoglobin A. In developing this model, we have studied factors which influence both hemoglobin exchange and restoration of low potassium permeability in resealed red cell ghosts, and we have arrived at a method which employs a useful compromise between these two somewhat mutually exclusive aims. The method allows preparation of hybrids which exhibit total hemoglobin replacement and potassium concentration near that of normal cells.

From the Departments of Medicine, Clinical Pathology, and Laboratory Medicine, Division of Hematology, University of California, San Francisco, Calif. 94143.

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Address for reprint requests: Stephen B. Shohet, M.D., Departments of Medicine, Clinical Pathology, and Laboratory Medicine, Division of Hematology and Cancer Research Institute, University of California, San Francisco, San Francisco, Calif. 94143.

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normal cells. Using these hybrids, we have demonstrated that membranes from normal cells which contain hemoglobin S will sickle reversibly upon deoxygenation. With prolonged deoxygenation, these hybrids sickle irreversibly. We suggest that erythrocyte hybrids will serve as a useful model for studying membrane alterations associated with the sickling process.

MATERIALS AND METHODS

Hybrid Preparation

The preparation of hybrids is an extension of Passow's methods for the preparation of resealed ghosts not containing hemoglobin.10,11 Fresh membrane donor cells are washed once in isotonic phosphate buffer at the desired lysis pH. The cells are then hemolyzed for 2 min at 0°C in 40 volumes of diluted phosphate buffer containing 300 mg/100 ml of dialyzed bovine serum albumin (BSA) and in some cases 1 mM EDTA (final osmolality of hemolysate, 40 mosmoles). Immediate centrifugation at 39,000 g for 2 min separates the membranes from the hemoglobin before rapid addition of the desired replacement hemoglobin. We then add three volumes of 25-30 g/100 ml hemoglobin concentrate to 1 volume of ghosts. All components are kept at 0°C, and the hemoglobin is oxygenated by swirling in air just prior to use. After allowing the membrane-hemoglobin mixture to equilibrate for 5 min, ATP (80 mM at pH 7) is added to a final concentration of 1 mM, along with any desired divalent metals or chelating agents. The solution is mixed, and enough 3.8 M KCl is added to restore osmolality to 290-295 mosmoles/kg. The amount of salt required is calculated on the basis of the volumes of the ghost suspension and hemoglobin concentrate, direct measurements of the osmolality of the hemoglobin before concentration, and the amounts of added ATP, divalent metals, or chelators. We also allow an additional 5 mosmoles for the effect of concentrating the hemoglobin. This somewhat complex approach is necessary because the osmometer cannot be used with concentrated hemoglobin and ghost suspensions.

After raising the osmolality of the membrane-hemoglobin mixture, we “anneal” the suspension at 37°C to complete resealing of the membranes around the newly introduced hemoglobin. Gentle resuspension and washing in iso-osmolar phosphate buffer or buffered saline with glucose plus 300 mg/100 ml BSA then removes excess untrapped hemoglobin, potassium, and other added substances.

Hemoglobin Preparation

Concentrated hemoglobin solutions are prepared from cells washed thoroughly in sodium phosphate buffer (pH 7.4, 291 mosmoles/kg). The cells are hemolyzed in 10 volumes of cold distilled water to achieve a final osmolality near 30 mosmoles/kg without excessive dilution of the hemoglobin. (If the osmolality of the hemoglobin after concentration is much higher than 40 mosmoles/kg, the ghosts do not incorporate hemoglobin adequately during hybrid preparation.) Following lysis, the hemoglobin is separated from the membrane fraction by two centrifugations at 27,000 g for 20 min. Freezing the dilute hemoglobin solution between the two centrifugations promotes aggregation and sedimentation of any suspended material during the second centrifugation. For scrupulous removal of any remaining ghosts, we pressure filter the hemoglobin through a DPO2 Diapore filter just before reconcentrating it to about 27 mg/100 ml on an Amicon PM10 ultrafiltration membrane. Usually, the hemoglobin is stored as a dilute solution.

*Isotonic phosphate buffer is prepared from monobasic and dibasic stock solutions adjusted to 290-295 mosmoles/kg using a Fiske osmometer. The stock solutions consist of approximately 22.3 g/liter NaH₂PO₄·H₂O and 34.7 g/liter Na₂HPO₄·7H₂O. Osmolalities are measured and readjusted if necessary. One stock solution is titrated with the other to achieve the desired pH. For example, 500 ml of Na₂HPO₄ requires about 34 ml of NaH₂PO₄ to obtain a pH of 7.4.

 Buffered saline with glucose following Corashe NaCl, 8.1 g; Na₂HPO₄·7H₂O, 2.302 g; NaH₂PO₄·H₂O, 0.194 g; glucose, 2.0 g, made up to 1 liter with distilled water. The pH and osmolality are finally determined and adjusted to 7.4 and 290-295 mosmoles/kg if necessary.
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Hybrid erythrocytes (1\textsuperscript{st} and 2\textsuperscript{nd}) frozen at -12°C and reconcentrated 1 day prior to "hybridization." Thawed hemoglobin solutions contain from 6\textsuperscript{o} to 10\textsuperscript{o} methemoglobin (calculated from spectral measurements at 560, 576, and 630 nm\textsuperscript{14} and this proportion of methemoglobin does not increase significantly during storage periods of 1 wk-3 mo. Resealing and sickling characteristics are the same in hybrids prepared with fresh hemoglobin and hemoglobin stored for up to 3 mo. However, storage of concentrated hemoglobin (>10 g/100 ml) or prolonged storage of 5-6 mo does result in unacceptably high levels of methemoglobin. The amount of methemoglobin also increases during the annealing of hybrids. The maximum cumulative levels in the final hybrids is 15\textsuperscript{o} to 20\textsuperscript{o} of the total hemoglobin; however, this level has not been sufficient to interfere with either the sickling of hybrids or the reversibility of sickling in fresh hybrids.

Separation of Low Permeability Hybrids

Annealed, washed hybrids are separated according to density on a discontinuous gradient of Stractan II, employing the methods of Corash\textsuperscript{12} with the following minor modifications: The Stractan solutions are deionized by mixing directly with Amberlite in a beaker (300 g Stractan to 450 g resin) rather than passing them through a column. After a 30-min equilibration period at 4°C, the Stractan solutions are separated from the resin by filtration. This deionization step is repeated with freshly regenerated resin until the osmolality of the Stractan is below 100. Regeneration of the mixed bed resin is preceded by separating the basic and acidic fractions by decanting the less dense anion-exchanging resin during several washes with distilled water. The anion exchanger is then regenerated with 10 volumes of 3 N NaOH, and the cation exchanger with 3 volumes of 3 N HCl (room temperature, 30 min). After thorough washing to neutral pH, the resins are recombined and stored at 4°C. The only other modification of the Corash procedure is the substitution of sodium phosphate (0.15 M, pH 7.4) for potassium phosphate as a buffer in the Stractan stock solutions.

Gradients are prepared by layering 1 ml each of four Stractan solutions with densities of 1.065, 1.057, 1.048, and 1.040, respectively, into a 5-ml tube for a Beckman SW 50.1 rotor. Up to 0.5 ml of hybrids suspended in buffered saline with glucose is layered on top of the gradient and then centrifuged at 50,000 g for 45 min. Hybrids at the interfaces are collected with a Pasteur pipet and washed three times in buffered saline with glucose plus 300 mg/100 ml BSA.

Cation Retention and Hemoglobin Exchange Determinations

To measure retention of potassium added at the time of resealing, duplicate hybrid samples are sedimented in microhematocrit tubes, and packed cell volumes, hemoglobin, and potassium are measured\textsuperscript{15,16}. The extent of hemoglobin exchange is assessed by hemoglobin electrophoresis of hybrid hemolysate preparations on cellulose acetate strips.

RESULTS

Sickling of Hybrids

Using the methods described above, we have found that it is possible to prepare hybrid erythrocytes from both normal and sickle membranes with either HbA or HbS. One can readily obtain MCHC of 16-19 g hemoglobin per 100 ml, which is sufficient to allow sickling of HbS containing hybrids. Such HbS hybrids sickle when deoxygenated with N\textsubscript{2} or Na\textsubscript{2}S\textsubscript{2}O\textsubscript{5}, whether their membranes are derived from normal or sickle cells. A scanning electron micrograph of normal membrane:HbS hybrids, sickled with sodium metabisulfite, is shown in Fig. 1. These hybrids appear very similar to native sickle cells under the same conditions, except for their decreased mean cell volumes. Conversely, hybrids containing HbA do not sickle under any conditions, regardless of the source of their membranes.
Fig. 1. Scanning electron micrograph of normal membrane:HbS hybrids deoxygenated with Na$_2$S$_2$O$_5$ before fixation. Cells were fixed according to Bessis and Weed.\textsuperscript{17} The figure is typical of multiple preparations deoxygenated with either Na$_2$S$_2$O$_5$ or N$_2$.

Cation Retention and Hemoglobin Exchange

Having shown that it is possible to prepare hybrids with the desired combinations of hemoglobin and membranes, we have studied properties which are crucial determinants of the hybrid’s usefulness as a model for membrane changes associated with sickling. The two most stringent requirements on the hybrid system are restoration of near-normal cation permeability after membrane resealing and complete hemoglobin exchange. It is clear that study of membrane permeability changes during the sickling process would be impossible if the resealed hybrids are excessively permeable. Further, to obtain a clear answer as to whether a particular aspect of membrane function is intrinsic to the membrane or is solely dependent upon the hemoglobin contained within the membrane, it is necessary to achieve total hemoglobin replacement. We have found that both cation permeability and hemoglobin exchange are sensitive to the pH and the presence or absence of divalent metals during the brief period in which the ghosts remain open.
Table 1. Hybrid Hemoglobin Exchange and Potassium Retention* as a Function of Lysis pH

<table>
<thead>
<tr>
<th>Lysis pH</th>
<th>Residual Membrane Donor Hemoglobin (% of total hybrid Hb)</th>
<th>Potassium Retention (%)</th>
<th>Potassium Concentration (meq/liter hybrids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2</td>
<td>14</td>
<td>70</td>
<td>80</td>
</tr>
<tr>
<td>7.0</td>
<td>-</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>7.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Potassium retention is defined as the percentage of the potassium concentration in the annealing mixture which remains in the hybrids after three washes in potassium-free buffer.

Table 1 summarizes the effect of the pH during lysis on overall hemoglobin exchange and the retention by hybrids of potassium added just before the annealing step. As anticipated from the work of Dodge and Passow on hemoglobin-free ghosts, the pH has opposing effects on the desired hybrid characteristics. Hybrids prepared with lysis at pH 7.4 exhibit total hemoglobin exchange. However, hybrids with sufficiently high potassium retention for use in cation flux experiments contain a high level of contaminating hemoglobin from the membrane donor cells. To obviate this problem, we add EDTA to the lysis medium, which, as shown by Bramley, promotes hemoglobin release from ghosts. With lysis at pH 6, in the presence of 1 mM EDTA, hemoglobin exchange is again virtually complete, with no residual membrane donor hemoglobin in the hybrids. This result is demonstrated by the hemoglobin electrophoresis scans shown in Fig. 2.

The presence of EDTA in the lysis medium reduces the overall hybrid retention of potassium to about 25%–35% of the potassium concentration in the annealing mixture, or approximately half the retention by hybrids prepared in the absence of EDTA. Assuming that this reduction is due to an increase in the proportion of hybrids that are totally leaky to potassium, we have then employed density gradient centrifugation in an effort to isolate a subpopulation of hybrids with low potassium permeability. Corash had shown that the arabinoxylan Stractan II provides a convenient and noninjurious supporting medium for separation of whole red cells according to density. As can be seen from Fig. 3, Stractan is also suitable for selection of the potassium-retentive cells from a heterogeneous mixture of leaky and resealed hybrids. In the typical experiments for which the potassium concentrations are tabulated, we were able to isolate subpopulations of high-potassium hybrids containing 53 and 98 meq/liter. These came from initial hybrid mixtures containing only 25 and 58 meq/liter, respectively.

Effect of Divalent Cations

Passow's work had previously demonstrated the importance of divalent metal cations in the resealing of ghosts. However, his experiments involved addition of Mg or Ca to the lysis medium, and we wished to avoid this because their presence in the lysis medium increased retention of hemoglobin by the membrane. Thus, we added Mg after lysis, along with the ATP, at final concentrations of 1.0 or 0.5 mM. More recently we have found that the addition of Ca at this point was more effective than Mg in the enhancement of resealing. This is demonstrated by Fig. 3, which shows Stractan gradient separations of
Fig. 2. Electrophoresis of hemoglobin from membrane and hemoglobin donor cells and resultant hybrids. The sickle cell blood used in both experiments contained a small amount of fetal hemoglobin which appears as a shoulder on the left of the peak for HBS. A comparison of the scans for donor cells with those for the corresponding hybrids shows virtually complete replacement of the membrane donor hemoglobin with that from the hemoglobin donor cells. (The electrophoresis was performed in the Moffitt Hospital clinical laboratory on cellulose acetate strips with a Beckman microzone apparatus, using a discontinuous buffer system. Tris-EDTA-borate buffer at pH 9.1 was used at the anode, and barbital buffer at pH 8.6 was used at the cathode. The strips were scanned with a Clifford densitometer.)

Fig. 3. Distribution of hybrid fractions on discontinuous Stracton gradients. Hybrids were resealed in the presence of either 1 mM Mg$^{2+}$ or 1 mM Ca$^{2+}$. Potassium and hemoglobin concentrations in each subpopulation are listed, along with the fraction of the total hybrids which that subpopulation represents. The hybrids illustrated were prepared from normal erythrocyte membranes and HbA.
hybrids resealed in a final concentration of 1 mM Mg and 1 mM Ca. Not only was the potassium concentration higher in each layer of the sample prepared with Ca, but the proportion of cells in the highly retentive layer was much greater than in the sample prepared with Mg. The relative distribution of hybrids in each layer was somewhat variable with the addition of Ca; the high K layer usually contained 80° - 90° of the hybrids, with K concentrations from 80 to 100 meq per liter cells, whereas, in the Mg preparations the high K layer contained only 40° of the cells with K concentrations varying from 50 to 90 meq per liter cells.

The hybrids illustrated were prepared from HbA and membranes from normal cells. Normal cell membranes with HbS produced equivalent results. How-

*With the exception of this step, all operations are performed at 0-4° C.

**Fig. 4. Schematic diagram of the hybrid preparation.**
Fig. 5. Phase-contrast photomicrographs of hybrids prepared in the presence of 1 mM ATP and 0.5 mM Mg. The hybrids were incubated under the conditions specified at 37°C in phosphate-buffered saline with 10 mM glucose and 0.3 g/100 ml bovine serum albumin, at approximately 4% hematocrit. (A) Normal membrane: HbS hybrids incubated for 17 hr in the presence of O2. (B) Normal membrane: HbS hybrids incubated for 17 hr in the presence of N2. (C) The same hybrids as in (B) reequilibrated with O2 for 30 min. (D) Sickle membrane: HbS hybrids incubated for 17 hr in the presence of O2. (E) Sickle membrane: HbS hybrids incubated for 17 hr in the presence of N2. (F) The same hybrids as in (E) reequilibrated with O2 for 30 min.

ever, hybrids prepared from sickle cell membranes retained only about half as much potassium as hybrids using normal cell membranes.

Resealing in sickle membrane hybrids was also enhanced by the presence of Ca; final hybrid K concentrations were about 50 meq/liter when Ca was used and about 30 meq/liter when Mg was used.

It should be noted that the resealing capacity of erythrocyte membranes
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appeared to be much less sensitive to Ca added after hemolysis than to the same concentrations of Ca in the hemolysis medium. For example, Passow found that maximum potassium retention by ghosts occurred with 0.01 mM Ca in the lysis medium and that with 1 mM Ca there was virtually no resealing. In contrast, in our system, 0.1 mM Ca added after hemolysis gave no enhancement of potassium retention, and 1 mM was optimal. Magnesium did not seem to promote resealing in hybrids with increasing concentrations up to 5 mM. Furthermore, in the presence of ATP, which we added to all the hybrids, Mg stimulated membrane endocytosis, as previously noted by others. The presence of Mg and Ca together, with ATP, further increased this effect. Thus, we added either Mg or Ca to the hybrids, depending on the purpose of the experiment, but we did not use both together.

The information derived from these several experiments has led to the present method for hybrid preparation, which is summarized schematically in Fig. 4.

Irreversible Sickling of Hybrids

After separation on the Stractan gradient, potassium-retentive hybrids containing HbS were incubated at 37°C for several hours in the absence of oxygen, then reequilibrated with oxygen for 30 min. A proportion of cells remained distorted and were morphologically indistinguishable from irreversibly sickled cells (ISCs). Figure 5 illustrates the morphologic changes in various types of hybrids during deoxygenation and subsequent reoxygenation. Hybrids containing HbS formed irreversibly sickled hybrids (ISHs) whether their membranes came from normal or sickle cells. (Expectedly, in experiments not illustrated, sickle membranes containing HbA could neither sickle nor form ISHs.) In the HbS preparation a few ISHs could be found after 4 hr of deoxygenation (5–10%). After 15–24 hr up to 80% of the sickled cells remained irreversibly sickled. Even after these lengthy incubations, the majority of parallel samples of HbS hybrids which had been incubated under oxygen would reversibly sickle upon deoxygenation; i.e., they were not "irreversible discocytes." In one experiment, we were able to sickle and unsickle a sample of hybrids five times on day 3 of continuous incubation, with little diminution in the ability of the non-ISH hybrids to sickle. Some additional methemoglobin was generated during prolonged O₂ incubation, and Heinz bodies formed, but there appeared to be no relationship between those processes and the presence or absence of irreversible sickling.

DISCUSSION

We have developed a method for the complete exchange of hemoglobin from one type of red cells with that of another. This exchange is accompanied by restoration of low membrane permeability to potassium. It has been stated frequently that the membrane leak for large molecules like hemoglobin, which appears during hypotonic hemolysis, is of very brief duration. To the contrary, we have found that under the low temperature conditions specified by Passow, a major fraction of the ghosts will remain open for at least 5–10 min. This phenomenon allows sufficient time for the removal of their native hemo-
globin before addition of the desired replacement hemoglobin, both minimizing
dilution and facilitating hemoglobin exchange.

Hybrids containing HbS exhibit important properties characteristic of whole
sickle cells, such as the capacity to sickle upon deoxygenation, to retain high
K⁺ concentration, and to remain permanently deformed after prolonged de-
ovoxyanation. We anticipate that the hybrid model will be useful for studying
the effect of sickling on the erythrocyte membrane because of two opportunities
it offers which are not available in whole cells. First, one can control the in-
ternal milieu of the membrane in hybrids; second, one can study sickling in
membranes which have never before been in contact with sickle Hb. The first
possibility allows introduction of factors which might either retard or accelerate
the degradation of membrane function which occurs with sickling (e.g., Ca²⁺ or
ATP). The ability to study the effect of sickling on membranes which have not
sickled before should tell us whether membrane degeneration attends the first
incident of sickling or whether it requires repeated sickling episodes. It should
be noted that there have been suggestions that membranes are altered during
the process of hemolysis and resealing. However, recent data indicate that
such changes may have resulted from the manner in which the membranes were
handled, rather than from the lysis and resealing procedures themselves.

Nevertheless, it is clear that the hybrid erythrocyte is different from a whole
normal cell in terms of cellular contents, and that as presently prepared it is
unlikely to be capable of normal metabolism. Thus, one cannot directly apply
results from experiments using the current hybrids to processes in vivo without
confirmatory experiments in whole cells. However, hybrids do offer the op-
portunity to assess, individually and in a controllable fashion, the influence of
diverse factors on the relationship between sickling and membrane function.

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