Evaluation of Covalent Antisickling Compounds by Po2 Scan Ektacytometry

By Robert M. Johnson, Claude J. Féo, Michael Nossal, and Irène Dobo

The ektacytometer, a device to measure erythrocyte flexibility, has been used to evaluate antisickling agents that covalently modify hemoglobin S (HbS). The instrument has been adapted to produce a continuous gradient of oxygen pressure in the measuring cuvette, which permitted the rapid determination of sickle cell rigidity over the complete oxygenation range. Inspection of curves allows classification of the compounds according to their mode of action: altering oxygen affinity or increasing deoxy-HbS solubility. Reagents that modify amino groups, thiol, and histidine, as well as a crosslinking agent, were examined. The method directly evaluates deoxygenated cell deformability rather than cell shape. Many of the compounds that are effective in preventing the morphological sickling of deoxygenated sickle cells do not necessarily restore cell deformability. The method also readily detects membrane damage brought about by covalent agents that nonspecifically derivatize membrane proteins. Cystamine and pyridoxal appear to improve deformability in deoxygenated SS cells at concentrations that do not damage the membrane. This method, which examines the intact cell, fills a gap in the available experimental techniques for drug evaluation, between studies of isolated hemoglobin and in vivo studies.

© 1985 by Grune & Stratton, Inc.

Materials and Methods

The ektacytometer has been described in a number of earlier publications. It consists essentially of a cylindrical clear plastic Couette viscometer, with the addition of an optical light path which directs the beam of a helium-neon laser through the cylinders in a direction normal to the axis of rotation. Red cells enter the viscometer, where they refract the laser beam, and the pattern of the diffracted light supplies information about the shape of the cells. The pattern can be evaluated visually or by means of a simple image analyzer. The deformation is expressed as the ektacytometric index, which ranges from 0 for undeformed discocytes to about 0.7 for maximally elongated cells.

To form a continuous O2 gradient, the following modifications were made. Two bottles of buffer in a 37 °C bath were gassed with either N2 or air. The gradient was made by two peristaltic pumps driven by stepping motors which gradually increased the proportion of air-gassed buffer in the stream in both the dead volume and the sample buffer. The blood sample, appropriately diluted and deoxygenated in a tonometer, was added to the mixed buffer stream by a third peristaltic pump, the "sampling pump." The cell suspension passed through a heating coil at 37 °C, whose length was adjusted to give a transit time of one minute. This permitted complete mixing and equilibration of the cells and the O2 gradient. The cells then entered the viscometer where O2 concentration was measured with a Clark electrode (BG Analyzer, Technicon Instr. Corp., Tarrytown, NY) installed in the outer cylinder. The outputs of the electrode and the image analyzer were fed into an X-Y recorder.
samples were obtained from individuals undergoing examination for hemoglobinopathies. The phenotype of all samples had been determined electrophoretically. For compounds that form covalent links, the cells were again washed and 150 μL (hematocrit of 40%) were mixed with 5.0 mL of deoxygenated PVP buffer in the tonometer under N2. After five minutes, the tube leading to the sample pump was introduced to begin the run. In all but a few cases in which sample volume was limited, control erythrocytes (incubated without the agent) were run at the end as well as at the beginning of a sequence of experimental curves, to verify that no change in instrumental characteristics had occurred. The figures show typical results, using a single donor sample for all curves. All compounds were tested with at least three different donor samples.

**Ektacytometry of Ghosts.** Erythrocytes (300 μL) were incubated with the agent as described above, at a hematocrit of 1%, for the times indicated in the figure legends. The cells were washed three times in PBS-10 mmol/L glucose and Po2 scans were run to verify that the expected alteration of deformability had occurred. White ghosts were prepared by lysis and washing in 5 mmol/L NaPi, pH 7.4, 0 °C. For fragmentation assays, the general procedure of Mohandas et al15 was followed. The ghosts were packed at 12,000 rpm (Sorvall SS34 rotor) for 15 minutes, resuspended in ten vol of PBS and resedimented by centrifugation and resedimented in 2.5 mL of 40% dextran in PBS. This suspension was introduced into the viscometer, and rotation was begun at 150 rpm. The initial EI is a measure of membrane flexibility, and the decline in EI with time measures membrane fragmentation.

**Data Analysis.** The deformability of sickle cells, as measured by the ektacytometer13:14, increases during oxygenation, with a sigmoid dependence on O2 concentration. Three features of the ektacytometric curves are used to characterize the effects of antisickling agents: 1. PE10, the Po2 at which 50% of the total observed change in EI occurred. This is not necessarily the same as Po50, the midpoint of the oxygen affinity curve, but is rather a measure of the relation between O2 and cell rigidity. 2. The EI10, the EI of completely oxygenated cells. It is a minimum criterion for an effective agent that the flexibility of cells with depolymerized hemoglobin should not be affected. 3. The EI50, the index under completely deoxygenated conditions. This value is determined primarily by the fraction of cells that have become rigid, but is also influenced by shape factors (Fig 1). The EI for rigid cells is 0, if the cells are symmetric.16 Asymmetric rigid cells such as deoxygenated SS or AS cells do not orient themselves with their long axes parallel to the viscometer axis.16 The resulting defraction pattern is turned 90° to the viscometer axis and contributes a negative EI signal, whose magnitude will depend on the detailed cell shape and its orientation. These factors are not well understood at the present time. Because of the contribution of rigid cell orientation to the index, we have chosen to use the term EI rather than D1 (deformability index) for work with sickle cells. The components of EI due to shape and deformability both increase as cells become flexible. It is clear that cell deformability has the major effect on the index, since the span of EI from fully deformable to rigid normal cells is approximately 0.6, while the greatest negative value we have observed in maximally polymerized AS cells was −0.18. In general, EImax differed somewhat between blood samples and depended on the exact experimental conditions. In order to compare results with different samples, we express the data as Δ EI:

\[
\Delta EI = \left( EI_{max} - EI_{mean} \right) / \left( EI_{max} - EI_{min} \right) \times 100.
\]

Compounds that inhibit sickling by increasing oxygen affinity shift PE10, while compounds that increase deoxy-HbS solubility decrease Δ EI.14 This distinguishes the two classes of agent.

**Effect of Cell Type, pH, and Osmolality.** Data analysis was more difficult for SS cells, because of their great heterogeneity. For example, the presence of irrevocably sickled cells introduces a constant negative EI signal,16 which is manifested by a decrease in EI throughout the O2 range. Hence EImax is always lower for SS cells. In addition, SS cells have a much wider range of mean corpuscular hemoglobin concentration (MCHC) values and their membranes show numerous secondary abnormalities. In order to demonstrate the effects of the agents on SS erythrocytes, the EImax of SS cells was increased by lowering the medium osmolality, thus hydrating the irreversibly sickled cell fraction,17 allowing them to deform.

For many experiments, we used AS cells from clinically normal donors. These cells have the advantage of a much narrower range of MCHC and a more normal cell shape and membrane than SS erythrocytes. In our hands, maximal rigidity, the lowest attainable value of EI, for AS cells could be induced at pH 7.0. The medium osmolality was routinely increased to 300 to 310 mosm, although this was not strictly necessary. To avoid possible nonequilibrium or kinetic effects, we determined that the conditions chosen induced the maximal degree of rigidity at Po2 = 0 mm Hg (ie, further lowering of the pH did not decrease EI) and that the process of cell stiffening was complete (deoxygenation periods longer than five minutes did not significantly change the curve of EI vs Po2).
RESULTS

Sulfhydryl Reagents

Cystamine caused a marked increase in the $O_2$ affinity of sickle cells. AS (Fig 2A and B) and SS (Fig 2C) erythrocytes were treated with increasing amounts of cystamine. The extent of derivation was not determined, but an estimate of 50% modification at 2 mmol/L can be obtained from the data of Hassan et al. Five mmol/L cystamine caused cell damage ($E_{i\text{max}}$ was lowered). Cystamine was very effective at submillimolar concentrations, at which no cell damage was noted. In addition to its major effect on PEI$_{50}$, cystamine increased $E_{i\text{max}}$ slightly, indicative of its weak effect on solubility.

Amino Group Reagents

Pyridoxal. A number of compounds able to form Schiff base adducts, primarily with $N$-terminal amino groups, have been suggested as antisickling agents. Some of these compounds, eg, pyridoxal or 5'-deoxypridoxal, are reported to increase $O_2$ affinity, whereas others, eg, pyridoxal sulfate or glyceraldehyde, act primarily to increase solubility. Because it readily enters unwashed cells to form covalent adducts, we chose pyridoxal as representative of the first class of aldehyde reagents. In AS cells (Fig 3A and B), pyridoxal did cause a slight leftward shift in PEI$_{50}$, indicative...
of an effect on O2 affinity, but unexpectedly, its major effect was to increase \( E_{\text{Min}} \). It is sometimes difficult to deoxygenate high-affinity Hb, and we have not documented that five minutes under \( N_2 \) was sufficient to completely remove the bound \( O_2 \) of pyridoxal treated Hb. It is therefore possible that the elevated \( E_{\text{Min}} \) could be attributed to incomplete deoxygenation. An elevated \( E_{\text{Min}} \) is, however, characteristic of drugs that increase deoxy-HbS solubility, and this explanation is consistent with recent reports that pyridoxal significantly increases the solubility of deoxy-HbS. Pyridoxal did not alter oxygenated cell deformability. The results for SS cells are qualitatively similar (Fig 3C).

**Glyceraldehyde.** Like pyridoxal, Schiff base adducts with this compound are reported to inhibit cell sickling. Unlike pyridoxal, it was reported to increase \( O_2 \) affinity only slightly, but to have a major effect on the solubility of HbS. As expected for an agent without a significant effect on oxygenation, ektacytometric analysis of AS erythrocytes treated with 10 and 20 mmol/L glyceraldehyde (Fig 4) showed no shift in the \( Pe_{150} \). Surprisingly, however, there was also no improvement in their deformability at low \( P_{O_2} \), and \( E_{\text{Max}} \) was reduced. We examined the morphology of glyceraldehyde-treated cells after 20 minutes of deoxygenation in the tonometer. The cells were fixed with 1% glutaraldehyde in PBS equilibrated with \( N_2 \). As reported earlier, treated cells did not change their shape when deoxygenated, ie, glyceraldehyde did prevent sickling. This suggested that glyceraldehyde induced a general reduction in cell flexibility, such that intracellular polymerization of deoxy-HbS did not result in morphologically sickled cells even though the cells were clearly rigid. Since osmotic fragility is not affected by glyceraldehyde, volume changes were not the cause of reduced erythrocyte deformability. Membranes from treated cells were therefore examined (Fig 5) and were found to have decreased flexibility, as manifested by a lowered initial \( E_i \). Mechanical stability was also greatly increased, as shown by the long half-times for ghost fragmentation.

**Histidine**

Nitrogen mustard is reported to modify histidine residues and to inhibit sickling. When AS or SS cells were treated with nitrogen mustard, results qualitatively similar to glyceraldehyde were obtained (Fig 6). \( E_{\text{Max}} \) was diminished, but \( E_{\text{Min}} \) was not improved. The flexibility of ghosts from treated cells was diminished, and their mechanical stability was markedly increased (Fig 7).

**Cross-linking Agents: Dimethyladipimidate (DMA)**

This reagent was reported to increase \( O_2 \) affinity and to prevent sickling, even of completely deoxygenated cells. It also increases deoxy-HbS solubility significantly. Ektacytometric scans (Fig 7) showed that DMA shifted \( Pe_{150} \) and lowered \( \Delta E_i \) as expected for an agent that alters both affinity and solubility. There was, however, a marked dimin-
JOHNSON ET AL

**Fig 7.** Fragmentation of ghosts from nitrogen mustard treated cells. Erythrocytes were incubated as described in the legend of Fig 6. Ghosts were prepared as described, and EI was determined as a function of time in a constant shear field.

**DISCUSSION**

For HbS, compounds that covalently modify lysine, cysteine, histidine, and N-terminal amino groups have been proposed as antisickling agents.\(^1\)\(^,\)\(^23\) We have recently reported\(^4\) on the use of the ektacytometer to classify and evaluate antisickling compounds. It was shown that the mode of action could be determined by an inspection of the EI vs. \(P_02\) curves. Agents that alter \(O_2\) affinity, such as cyanate, shift \(PEI_50\), whereas agents that increase HbS solubility, such as butylurea, raise \(EI_{min}\) and diminish \(\Delta EI\). The results reported in this paper suggest that cystamine and, to a lesser degree, pyridoxal are effective covalent agents that do not adversely affect erythrocyte flexibility. Cyanate is also effective.\(^3\)\(^,\)\(^4\) Other compounds, including nitrogen mustard, glyceraldehyde, and DMA, which are effective in preventing sickling, did not necessarily increase the deformability of deoxygenated AS or SS cells to the level of oxygenated cells. Indeed, the flexibility of these cells in \(O_2\) was reduced, apparently by increased membrane rigidity, since the flexibility of ghosts from treated cells was reduced. It is possible that the decreased oxygenated deformability noted after treatment might be due to increased cytoplasmic viscosity, but no effect on hemoglobin viscosity has been observed in solution studies with these agents. It should be noted that Lubin et al\(^27\) suggested the possibility of a membrane effect for DMA.

It is not unexpected that nonspecific covalent agents such as glyceraldehyde, nitrogen mustard, or DMA would modify the cell membrane. The converse finding, that certain agents (pyridoxal, cystamine) do not compromise membrane function even though they effectively alter HbS, is perhaps more surprising. An effective covalent agent will require stereochemical selectivity directing it to unique sites in the Hb molecule to avoid membrane alterations, since the membrane appears to be more susceptible to damage than is the overall metabolism of the cell. DMA, for example, did not affect glycolysis\(^27\) at concentrations that made the cell completely indeformable. An approach to stereoselectivity has been made with noncovalent agents designed to fit the intermolecular contact site in the fiber.\(^29\) The value of even a slight increase in selectivity can be seen by comparing glyceraldehyde, which derivatizes multiple sites of Hb,\(^29\) with pyridoxal, which is specific for the N-terminal valine of the \(\alpha\)-chain.\(^29\)\(^,\)\(^30\) The relatively nonspecific glyceraldehyde reacted with the membrane in a way that reduced its flexibility, while pyridoxal did not cause such damage. Early experiments with \((^{14}C)\)glyceraldehyde\(^23\) did in fact show substantial incorporation into the erythrocyte membrane.

Our failure to note an improvement in deoxygenated cell rheology with some of these agents contrasts with some earlier reports. For example, Nigen and Manning\(^29\) observed that glyceraldehyde treatment improved the bulk viscosity of deoxygenated SS cells at 40% hematocrit in PBS. The forces acting on the cells in this technique are not known precisely, since the deformation is caused by cell–cell collisions. It is highly probable, however, that the forces acting to deform deoxygenated sickle cells in the bulk viscosity experiment are much greater than those which we obtain in the ektacytometer. In the high-viscosity media used in the ektacytometer, erythrocytes act like liquid droplets,\(^31\) and deformation is

---

**Fig 8.** Effect of DMA. SS blood was incubated with DMA as described.\(^27\) Cells were washed in PBS with 10 mmol/L glucose. One volume of cells at 20% hematocrit in PBS-glucose was added to four vol of 0.14 mmol/L Tris-hydroxymethylamine, pH 8.80, with NaCl added to make 290 mosm, at 37 °C. Dry DMA (Pierce Chemicals) was added to make the indicated concentrations, and the cells were incubated at 37 °C for ten minutes. The erythrocytes were then washed three times with PBS-glucose, and the ektacytometric curves (A) were obtained in PVP buffer, pH 7.2, 290 mosm. (B) \(PEI_{50}\) and \(\Delta EI\).
dependent on a viscosity difference between the medium and the interior of the cell. The El will tend to go to 0 when internal viscosity exceeds the viscosity of the medium. A estimate of the internal viscosity of the deoxygenated sickle cell can be obtained from the work of Chien et al. At the shear rates used by Nigen and Manning (>100/s), the viscosity of a 35% HbS solution can vary from 10 centipoise (cP) at 100% O2 saturation to 100 cP at 67% O2 saturation. Further deoxygenation led to higher cP values, which Chien et al were not able to quantitate. Much of this range of internal viscosity was not detected in our experiments, since the medium viscosity was 12 cP. In the bulk viscosity experiments of Nigen and Manning, however, with greater deforming forces, variations of internal viscosity in the range of 100 cP or greater might have been detected. Since glyceraldehyde did not lower the bulk viscosity of deoxygenated SS cells to the level of oxygenated cells, it is likely that the treated cells had some internal viscosity between that of untreated deoxygenated cells and oxygenated cells. This is consistent with our data, which show that glyceraldehyde-treated deoxy-SS erythrocytes have a sufficiently elevated cytoplasmic viscosity to appear rigid in the ektacytometer. Similar considerations apply to the data of Roth et al. with nitrogen mustard. It is important to keep in mind the features of the erythrocyte which are examined in different procedures. Membrane rigidity and cytoplasmic viscosity both contribute to bulk viscosity, and the technique could not detect an increase in membrane stiffness if cytoplasmic viscosity was also changing. The ektacytometer permits these two factors to be separated, but is insensitive to changes in internal viscosity in the high cP range.

Cytoplasmic viscosity is the major determinant of sickle cell deformability, and probably of in vivo life span as well. However, some of the covalent agents increased the mechanical stability of the membrane threefold or more (Figs 5 and 7), which suggests the possibility that increased membrane resistance to shear fragmentation may be a factor in the improved in vivo survival of treated sickle cells. For example, studies of genetically fragile erythrocytes show that the rate of ghost fragmentation as determined in the ektacytometer is a good predictor of the ability of such erythrocytes to survive in the circulation. The rapid hemolysis of fragile erythrocytes is caused in part by their assumption of a spherical morphology followed by splenecy, but membrane fragility per se is also important. In the autosomal dominant form of hereditary spherocytosis (HS), for example, membrane mechanical stability is normal and the erythrocyte life span is relatively normal. Moreover, HS cell life span is normalized by splenectomy, even though sphericity is unchanged. Splenectomy is not curative in those anemias characterized by a fragile membrane. It is probable that increased deoxy-HbS solubility after nitrogen mustard treatment is the major factor improving in vivo survival of these cells, but the possible significance of the greatly augmented membrane resistance to shear fragmentation merits investigation.

The ektacytometric technique described here can play a unique role in the evaluation of antisickling agents. In vitro testing will logically begin with an examination of the effect of the proposed compounds on the properties of HbS itself. Chang et al recently published an extensive and valuable compilation of data using these procedures. The effect of proposed compounds on the intact erythrocyte should then be determined, but the methods at the cell level are much less well developed. Most published drug evaluations have relied on the quantitation of the number of deoxygenated cells that appear “sickled” upon microscopical examination. This morphological assay requires a great amount of labor to examine a range of O2 tensions or solution conditions, and can also be misleading, since it is not safe to assume that intracellular deoxy-HbS polymerization is always accompanied by cell sickling. Bessis et al have shown that birefringent polymer formation can occur in sickle erythrocytes that remain morphologically disoid. Similarly, oxygenated sickle cells whose deformability is reduced by dehydration or by glucose depletion do not change shape upon deoxygenation. After glyceraldehyde or nitrogen mustard treatment, there is a decrease in the intracellular viscosity of deoxygenated sickle cells, since deoxy-HbS solubility is increased. There is also an increase in membrane rigidity (Fig 5,6). The combination of these factors appears to be adequate to prevent gross morphological change in treated cells, even though the internal viscosity of the deoxy-SS cells is not restored to the level of the oxygenated cell. In contrast with the sickling assay, the ektacytometric technique requires only a few minutes, the cellular response to the complete P02 range is obtained, and perhaps most significantly, the physiologically relevant feature of the sickle cell is measured, ie, its rigidity. Membrane alterations also be distinguished from changes in internal viscosity. The disadvantages of the method lie in the necessity for a high-viscosity medium containing PVP or dextran, as well as its insensitivity to high intracellular viscosities. The sensitivity could be increased, however, by augmented solution viscosity. We have used unphysiological values of pH and tonicity to maximize the response of the sickle cell in the experiments reported here, but that is not a requirement of the method and other values can be used.

Non-morphological techniques to determine intracellular polymerization are difficult to quantitate (electron microscopy, fiber x-ray diffraction, cell birefringence), or not readily automated (filtration or bulk viscosity). A recently developed NMR method determines the extent of polymer formation in a large population of cells. It is not immediately obvious how much intracellular polymer is required to induce cell rigidity, and a correlation between ektacytometric and NMR measurements would be of great interest. None of these methods has had much application in drug evaluation. For the moment, ektacytometry appears to be the most convenient way of measuring the physiologically relevant property of cell rigidity in a large population of sickle erythrocytes.

ACKNOWLEDGMENT

The authors wish to thank Drs Rosa, Beuzard, and Galacteros (Hôpital Henry Mondor, Créteil) and Drs Labie and Wacjman (Hôpitaux Cochin, Paris) for allowing us to study the blood from their patients. We have appreciated stimulating discussions with Dr P. Bromberg, and the assistance of Mrs R. Perrin.
REFERENCES

32. Roth EF, Kaul DK, Fabry ME, Nagel RL: Nitrogen mustard is effective in improving red cell life span in sickle cell anemia patients, in: Workshop on Development of Therapeutic Agents for Sickle Cell Disease. Lister Hill Center, National Institutes of Health, Bethesda, Md, May 1983, p 28
Evaluation of covalent antisickling compounds by PO2 scan ektacytometry

RM Johnson, CJ Feo, M Nossal and I Dobo