Inhibition of Erythrocyte Sickling In Vitro by Pyridoxal

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ABSTRACT To test the antisickling activity of pyridoxal, we compared the oxygen affinity and the percent sickling at low Po2 of untreated erythrocytes with values for cells from the same blood sample incubated with pyridoxal, glyceraldehyde, or pyridoxine. Pyridoxal increased oxygen affinity much more than glyceraldehyde. 20 mM pyridoxal and glyceraldehyde had equivalent antisickling activity. At Po2 levels above 20 mm Hg, both agents reduced sickling to less than 2%. In samples examined by electron microscopy, pyridoxal reduced the percent sickled cells and the percent cells that contain hemoglobin S fibers by the same amount (from 74 to 3%). Pyridoxine had no effect on oxygen affinity or sickling. Pyridoxal reacts with intracellular hemoglobin to increase oxygen affinity, which inhibits hemoglobin S polymerization and sickling.

INTRODUCTION

Erythrocyte sickling is initiated by the polymerization of deoxyhemoglobin S. Although several derivatives of pyridoxal react with hemoglobin (Hb)¹ and inhibit polymerization of Hb S (1–3), none appear promising for clinical use because they are either B₈ antagonists (e.g., deoxypyridoxal) or they contain polar side chains which limit entry into the erythrocyte (e.g., pyridoxal phosphate). In contrast, pyridoxal is a form of vitamin B₈ which is actively transported into erythrocytes (4). However, pyridoxal appeared unreactive (5) or reacted so slowly with a dilute solution of Hb S (3) that it was not tested for antisickling activity. Only 10% of Hb S was modified after erythrocytes were incubated with 3 mM pyridoxal (6). In this experiment a large increase in oxygen affinity was noted. This suggested to us that pyridoxal did react extensively with Hb, but the adduct was dissociated in preparing hemolysates for electron focusing. We therefore examined the effect of pyridoxal on the oxygen affinity of intact erythrocytes and on the percent sickled cells that result from exposure to low Po2. Pyridoxal was compared to glyceraldehyde, another carbonyl compound which had defined antisickling activity (7, 8), and with pyridoxine, the alcohol analogue of pyridoxal.

METHODS

Materials. Pyridoxal hydrochloride, pyridoxine hydrochloride, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Heparin without preservative was obtained from Flow Laboratories, Rockville, Md. All other chemicals were reagent grade.

Preparation of treated erythrocytes. Venous blood was drawn with informed consent from donors who had a normal Hb phenotype (AA) or a phenotype consistent with homozygous Hb S (SS), (9). For investigation of antisickling effects, blood was drawn into anticoagulant citrate phosphate dextrose solution (CPD) and stored at 4°C for up to 6 days. Erythrocytes were washed three times and suspended at 10% hematocrit in phosphate buffer that consists of 145.4 mM NaCl, 4 mM KCl, 1.65 mM Na₂HPO₄, 0.16 mM KH₂PO₄, 11.1 mM d-glucose, and 50 mg/liter bovine serum albumin. Four reaction mixtures were prepared from each suspension; a control and mixtures with 20 mM pyridoxal, glyceraldehyde, or pyridoxine. The pH was adjusted to 7.4 and each mixture was adjusted to 290±2 mosmol, excluding the compound tested. Reaction mixtures were incubated in a shaking water bath at 37°C in dim light for 90 min, transferred to shaking tonometers, and equilibrated for 30 min at 37°C with a humidified mixture of N₂, O₂, and CO₂. The partial pressure of each gas was varied independently, with an Ohio Medical Products anesthesia machine with modified fittings (Ohio Medical Products Div., Madison, Wis.). Before and after equilibration, the pH and PO₂ were measured in a Corning 161 Blood Gas Analyzer (Corning Medical, Corning Glassworks, Medfield, Mass.) and cells were drawn anaerobically into cold 1% buffered glutaraldehyde at pH 7.4. To eliminate the effect of CPD or buffer on erythrocyte oxygen affinity, this ex-

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¹Abbreviations used in this paper: AA, normal hemoglobin phenotype; CPD, anticoagulant citrate phosphate dextrose solution; Hb, hemoglobin; Fₛₒₜ, the Po₂ at which hemoglobin is half saturated; SS, homozygous hemoglobin S phenotype.
Experiment was repeated with fresh, heparinized whole blood from four SS patients.

Effects on oxygen affinity. Fresh, heparinized whole blood from two AA and two SS individuals was incubated and exposed to low Po2 as described above. The percent oxy-Hb was measured with an IL 182 Co-Oximeter (Instrumentation Laboratory, Inc., Lexington, Mass.) to define the linear portion of the oxygen dissociation curve for each reaction mixture. The Po2 at which Hb was half saturated (P50) was calculated from a linear regression.

Effects on sickling. Two observers counted from 250 to 500 fixed erythrocytes from each reaction mixture with an AO H21 interference microscope (C. Reichert, sold by American Optical Corp., Scientific Instrument Div., Buffalo, N.Y.) with a ×100 oil immersion objective. The observed variance of the proportion of sickle cells in each sample (as defined by counting subgroups of 50 cells) was close to that predicted by the binomial distribution. Slides with greater variation were prepared again. The number of cells counted was chosen to identify a 20% decrease in sickling with a false negative rate <0.05 and a false positive rate <0.01 (10). Erythrocytes were considered reversibly sickled if the cells were spiculated and elongated or had a transparent veil of cytoplasm along one side (11, 12).

Fixed erythrocytes from 20 experiments were also examined and photographed in an AMR-1000 scanning elec-

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**FIGURE 1** Effect of three compounds on the oxygen affinity of whole blood. Points on the linear part of the oxygen dissociation curve were determined after incubation of whole blood untreated or with 20 mM compound added: (a) a single AA blood sample; (b) a single SS blood sample.

**FIGURE 2** Effects of three compounds on erythrocyte sickling at varied oxygen tensions. Erythrocytes were incubated 90 min at 37°C, untreated or with 20 mM compound, equilibrated at low Po2 for 30 min, fixed, and examined. (a) Studies of cells from eight SS patients with stored, washed cells resuspended in buffer as described in Methods. (b) Studies of fresh, whole SS blood from four patients.

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RESULTS

Effects on whole blood oxygen affinity. The effects of pyridoxal, glyceraldehyde, and pyridoxine on the oxygen affinity of whole blood are shown in Fig. 1 and analyzed in Table I. Pyridoxal produced a much greater left shift in the oxygen dissociation curves than glyceraldehyde. Pyridoxine had no effect on oxygen affinity.

Effects on erythrocyte sickling. The relation between percent sickling and P₅₀ was determined for erythrocytes drawn in CPD and suspended in buffer (Fig. 2a). Pyridoxine had no effect on sickling. Plots for pyridoxal and glyceraldehyde were identical, with significant reduction in sickling (pyridoxal, P < 0.001; glyceraldehyde, P < 0.03, the sign test). In paired experiments on blood from five patients, the antisickling activity of pyridoxal (percent sickling, mean±SD, 7.8±6.4%) and glyceraldehyde (19.2±20.4%) were not significantly different (P < 0.3, paired t test). The relation between percent sickling and P₀₂ was not altered by use of heparinized whole blood (Fig. 2b) instead of erythrocytes in buffer. The following values were observed for blood from four SS patients treated with 5 mM instead of 20 mM pyridoxal: 0.6% sickling at a P₀₂ of 35.2 mm Hg, 1.3% at 26.3 mm Hg, 42% at 23 mm Hg, and 64% at 15 mm Hg. Sickling of untreated samples was 20, 81, 65.5, and 79.3%, respectively.

Scanning electron micrographs of representative samples (Fig. 3) confirmed the differences in degree of sickling noted by interference microscopy.

Effects on Hb S fiber formation. In transmission electron micrographs, untreated samples had a much higher proportion of cells that contain intracellular fibers characteristic of Hb S polymers (12) than did pyridoxal treated samples. A close correlation was found between the percent cells that contain Hb S fibers and the percent sickle forms by light microscopy (untreated cells: 75% Hb S fibers, 72.6% sickled; pyridoxal treated cells: 2.9% Hb S fibers, 2.4% sickle forms) (Figs. 3 and 4).

DISCUSSION

This report provides evidence that pyridoxal inhibits sickling of SS erythrocytes by modification of Hb S. Pyridoxal reduced the fraction of cells that contain Hb S fibers at low P₀₂ from 75% to 3%. A close correspondence was found between the percent sickled cells and the percent cells that contain Hb S fibers. Pyridoxal

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TABLE I

<table>
<thead>
<tr>
<th>Agent</th>
<th>P₅₀ (AA blood)</th>
<th>P₅₀ (SS blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>29.5</td>
<td>33.1</td>
</tr>
<tr>
<td>Pyridoxal, 20 mM</td>
<td>9.9</td>
<td>11.4</td>
</tr>
<tr>
<td>Glyceraldehyde, 20 mM</td>
<td>25.7</td>
<td>28.6</td>
</tr>
<tr>
<td>Pyridoxine, 20 mM</td>
<td>30.6</td>
<td>—</td>
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</tbody>
</table>

The P₅₀ was calculated from the linear regressions shown in Fig. 1.

FIGURE 3 Effect of pyridoxal on erythrocyte sickling. Washed erythrocytes from a single SS blood sample were suspended in buffer, incubated untreated or with 10 mM pyridoxal, equilibrated at a P₀₂ of 23 mm Hg, and fixed. Scanning electron micrographs at ×2,000 magnification show: (a) untreated cells with about 70% spiculated sickle forms; (b) pyridoxal treated cells with more than 90% discocytes, occasional echinocytes, and about 1% sickle forms.
activity of pyridoxal may be equivalent to the activity of these three agents and greater than that of cyanate (6).

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REFERENCES


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