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ABSTRACT

A new approach to the prevention of sickling in vitro using the bifunctional cross linking reagent, dimethyl adipimidate, is described. Sickling induced by anoxia can be totally prevented by a ten minute exposure of sickle erythrocytes to dimethyl adipimidate. Treated erythrocytes do not demonstrate the potassium loss and viscosity increase which usually accompany sickling. The oxygen affinity of hemoglobin in these cells is increased independently from changes in their concentration of 2,3 diphosphoglycerate. Since sickling is prevented in completely deoxygenated cells, the changes in hemoglobin oxygen affinity alone can not explain the mechanism of this effect.
INTRODUCTION

Recently, several attempts to inhibit sickling by modification of the hemoglobin molecule have been successful in vitro. Nitrogen mustard has been reported to inhibit sickling by a direct effect on hemoglobin gelation(1). In contrast, cyanate appears to act by modifying hemoglobin oxygen affinity(2). Preliminary clinical trials suggest that cyanate may be therapeutically beneficial in sickle cell disease (3), but potential side effects exist(4).

In this report, we describe a new anti-sickling agent, dimethyl adipimidate (DMA). This material is a bifunctional cross linking reagent which is known to covalently link the epsilon amino groups of lysine in polypeptides(5). At a concentration of 5 mM it effectively inhibits sickling and appears to have few deleterious side effects in vitro.

METHODS AND MATERIALS

Fresh heparinized blood samples were obtained from patients with sickle cell anemia. The cells were washed with Krebs Henseleit Buffer, pH 7.4, which contained 200 mg. % glucose and resuspended to a final hematocrit of 20%. To 4 ml. of the red cell suspension was added 16 ml. of 0.14 M tris-hydroxymethyl methylamine propane sulfonic acid buffer, previously adjusted to pH 8.8 with 5 N NaOH.

The entire suspension was then incubated for 10 minutes in a flask containing fresh, dry, powdered DMA at a final concentration of 5 mM. Control samples were prepared in an identical fashion except

* Obtained from Pierce Chemical Co., P.O. Box 117, Rockford, Illinois 61105
sucrose, (26 mM final concentration) was substituted for DMA. The osmolarity of the incubate mixture was 280 mosm. Following incubation, the cells were washed with Krebs Henseleit Buffer and resuspended in the original plasma at a final hematocrit of 40% and equilibrated with room air.

The number of sickle forms was measured after the preparations were deoxygenated by equilibration with nitrogen for 1 hour in an IL tonometer Model #237. Following equilibration, aliquots were anaerobically transferred into 10% formaldehyde in saline for fixation. 500 cells were counted using previously described criteria (6).

The viscosity of DMA treated and control cells was measured in a Wells Brookfield cone plate microviscometer Model LVT adapted to maintain controlled atmospheric gas tension (7). Following adjustment of the hematocrit to 40 ± 1%, the blood was equilibrated for one hour with humidified 95% N₂ and 5% CO₂ in an IL tonometer Model #237, then transferred in a closed system to the viscometer. The viscosity was measured at shear rates ranging from 4.5 to 90 sec⁻¹ during continued exposure to nitrogen.

In vitro studies of erythrocyte metabolism and of net K⁺ loss were conducted by incubation at 37°C of thrice washed erythrocytes in Krebs Henseleit Buffer with added glucose (10 mM) in the presence of 10⁻⁴ ouabain. The samples were continuously equilibrated with moistened gas mixtures consisting of nitrogen plus either 20%, 3.3%,
or 0% oxygen and pH was maintained at 7.45 ± 0.05 by varying the CO₂ concentration utilizing a gasometric pH stat (8). Supernatant K⁺ concentration was determined by flame photometry. Red cell 2,3 diphosphoglycerate (9) concentrations were determined in perchloric acid extracts.

The whole blood oxygen affinity, expressed as p50, (that oxygen tension at which hemoglobin is half saturated), was determined by measurement of hemoglobin oxygen saturation and pO₂ after equilibration of the samples with gas mixtures containing 2.2%, 2.8%, 3.2%, and 3.9% oxygen plus 5% CO₂ and the remainder nitrogen. The p50 was calculated by a best fit analysis of these data and corrected to a pH of 7.4.

RESULTS

The influence of DMA on the number of sickle forms following deoxygenation is shown in Figure I. The percentage of sickled cells was reduced from 80% in untreated cells to 4% by 5 mM DMA and to 0% by 10 mM DMA. The pH remained stable at 7.4 during these incubations.

The inhibition of sickling by DMA was further evaluated by measurement of net K⁺ loss from sickle cells incubated under hypoxic conditions. As previously shown, a marked increase in net K⁺ loss accompanies sickling (10). Determination of net K⁺ loss is, therefore, a quantitative measure of the amount of sickling (11,12). Ouabain was
added to the incubated cells in order to inhibit the ATP mediated cation pump and thus magnify the K+ loss induced by sickling. When sickling was absent, a small loss of K+ nevertheless occurred, due to the presence of ouabain. K+ loss was greatly accentuated by hypoxia, reflecting active sickling of erythrocytes. 5 mM DMA completely eliminated the net K+ loss induced by hypoxia in sickle cells. A representative experiment is shown in Figure II. In this experiment, 3.3% oxygen was chosen to induce a physiological level of hypoxia. Treated sickle cells incubated at 3.3% O₂ (closed circles) show net K+ loss within the usual range of 20% O₂ incubations where sickling is not observed. These results were not due to prior depletion of intracellular K+ by DMA since intracellular cation concentrations were unaffected by DMA. (Data not shown)

The whole blood viscosity of deoxygenated sickle cells was reduced at all shear rates by treatment with DMA. (Compare closed circles versus closed squares in the representative experiment which is shown in Figure III.) In contrast, the viscosity of deoxygenated normal blood which is less than that of sickle blood at similar shear rates, was slightly increased following DMA treatment. (Compare open circles and squares) However, it should be noted these slight changes in normal cells are still within our normal limits for this determination.

The oxygen affinity of sickle and normal cells was consistently and significantly increased following treatment with 5 mM DMA.
(Figure IV) The mean p50 value for whole blood for six patients with sickle cell anemia was decreased from $33.0 \pm 1.7$ mm. Hg. to $24.1 \pm 0.56$ mm. Hg. In five normal controls, the p50 value was decreased from $28.7 \pm 1.4$ mm. Hg. to $23.5 \pm 1.4$ mm. Hg. Erythrocyte 2,3 diphosphoglycerate levels in DMA treated sickle cells were slightly lower ($5.69 \pm 0.5$ mM/L cells) than in sickle cells not treated with DMA. ($5.90 \pm 0.23$ mM/L cells). Changes of similar magnitude were produced in control erythrocytes. In neither sickle nor control red cells were the differences in red cell 2,3 diphosphoglycerate large enough to account for the changes observed in hemoglobin oxygen affinity (13).

Preliminary studies with sickle erythrocytes have revealed that 5 mM DMA does not substantially alter glucose consumption, lactate production or glycolytic red cell enzyme activity. Red cell indices were altered slightly by DMA. A 5% increase in the mean corpuscular volume and a 5% decrease in the mean corpuscular hemoglobin concentration occurred in both normal and sickle cells, while the mean corpuscular hemoglobin remained unchanged.

**DISCUSSION**

In this paper, we present evidence which indicates that an 8 carbon bifunctional cross linking reagent, DMA, 

\[
\text{NH}_2^+ \quad \text{NH}_2^+ \\
\text{CH}_3\text{O-C-}(\text{CH}_2)_4\text{C-CH}_3
\]

is a potent inhibitor of sickling. Like cyanate, another inhibitor of
sickling, DMA alters hemoglobin oxygen affinity. Unlike cyanate, DMA inhibits sickling even under conditions of complete deoxygenation, indicating that factors other than altered oxygen affinity must also be involved.

Previous studies have indicated that bifunctional cross linking reagents may alter the structure of proteins without affecting their functional properties. For example, when ribonuclease is treated with DMA, enzymatic function is unaltered despite intrachain cross linking between the epsilon amino groups of lysine residues(14). In human erythrocytes, Neihaus and Wold have demonstrated that concentrations of DMA as low as 0.8 mM covalently cross link lysine residues in both the membrane and hemoglobin. This cross linking had no adverse effect on glucose consumption by intact cells and inhibited the release of oxygen from hemoglobin (5). Krinsky, Bymun and Packer have demonstrated that human erythrocytes treated with DMA are resistant to osmotic lysis but maintain their responsiveness to gramicidin induced K+ permeability (15). This suggests that a physiologic stabilization of the membrane may have been induced by this agent. Taken together with the effects on oxygen affinity, these results indicate that a combined effect on both the membrane and hemoglobin may account for the inhibition of sickling by DMA.

It is premature to speculate on the possible clinical role for DMA in the treatment of sickle cell disease. However, both the marked inhibition of sickling and the apparent lack of major effects on
erythrocyte metabolism, suggest that this agent might be used without seriously disturbing normal red cell physiology. Any therapeutic application must await delineation of the mechanism whereby sickling is inhibited and a thorough evaluation of possible toxicity.
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REFERENCES


LEGENDS

Figure I. Influence of DMA on sickling.
Following a 10 minute treatment with varying concentrations of DMA, the cells were equilibrated with nitrogen for one hour and fixed in formalin. The percent sickled forms was determined by counting at least 500 cells (6).

Figure II. The affect of DMA on net K+ loss from hypoxic sickle cells.
The range of net K+ loss from sickle cells following equilibration with either 3.3% or 20% O₂ is indicated by the shaded areas. A representative experiment in which sickle cells were treated with 5 mM DMA and then equilibrated at 3.3% O₂ is shown by the solid circles. Net K+ loss from the same cells not treated with DMA is shown by the open circles. 10⁻⁴ ouabain was present in all incubations.

Figure III. Viscosity of DMA treated erythrocytes.
Following treatment with either 5 mM DMA or 26 mM sucrose, the whole blood was equilibrated with nitrogen for one hour and the viscosity measured in a cone plate viscometer at varying shear rates. The hematocrit of all samples was 40 ± 1%.

Figure IV. The influence of DMA on the oxygen affinity of hemoglobin.
The oxygen affinity of hemoglobin in sickle cells and normal cells was measured before and after treatment with 5 mM DMA. The mean for each group is indicated by the horizontal bars. 2,3 diphosphoglycerate levels did not change significantly. (See text)
Fig. 1.

Percent sickle forms vs. mM concentration.
Fig. II.
Fig. III.
Fig. IV.
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