NITROGEN MUSTARD: AN "IN VITRO" INHIBITOR OF ERYTHROCYTE SICKLING

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SUMMARY - Sickling of erythrocytes from patients with sickle cell anemia can be completely inhibited by exposure to nitrogen mustard (HN_2). Reaction of hemoglobin (Hb) S with increasing quantities of HN_2 produces a progressive rise in the minimum concentration of hemoglobin required for gelation on deoxygenation. Quantities of HN_2 sufficient to alter gelation cause no observable change in oxygen affinity or in heme:heme interaction. Preliminary studies of osmotic fragility and autohemolysis showed no abnormalities after erythrocytes were treated with HN_2. Due to the known toxicity of HN_2, no "in vivo" intravenous therapy with this agent can be considered.

Sickle cell disease is characterized by the gross deformation of red cells upon exposure to low oxygen tensions. From the molecular point of view, sickling is the result of a single amino acid substitution near the N terminal residue of the beta chain of hemoglobin (Hb) S which enables the molecule to polymerize when deoxygenated (1). Many details of the sickling mechanism, such as the location of the binding sites, the types of bonds involved and the macromolecular structure of the polymer are not fully understood. Nevertheless, it seems that more than one pair of binding sites is required (2), and it is very likely that in addition to hydrophobic bonds, electrostatic (3), and possibly hydrogen bonds are involved in the protein-protein interactions that result in sickling. Accordingly, it would seem reasonable to attempt to inhibit sickling by altering the surface charges of the hemoglobin molecule. This preliminary report describes the in vitro inhibition of sickling by an alkylating agent, nitrogen mustard (HN_2).

METHODS

Venous blood, anticoagulated with either citrate or EDTA, as indicated, was

obtained from patients with sickle cell anemia. Hemolysates were prepared by the method of Drabkin (4) and all samples were examined by electrophoresis on starch gel at pH 8.6 and agar gel at pH 6.4 (5) for the presence of Hb A or other variants. The maximum amount of Hb F in hemolysates was 5%, measured as the proportion of hemoglobin resistant to alkaline denaturation.

To test the effect of HN_2 on sickling of intact red cells, varying amounts of HN_2 (K and K Laboratories, Plainview, N.Y.) dissolved in isotonic phosphate buffered saline solution (PBS) were added in a volume ratio of 1:1 to either whole blood or to red cells washed three times in PBS. After incubation for two hours at 37°C, the mixtures were deoxygenated by equilibration in an atmosphere of 95% N_2-5% CO_2 or by the addition of equal volume of a solution of 2% Na metabisulfite. An aliquot of cells was then fixed in 2% glutaraldehyde with a 0.1 M Cacodylate-buffer and evaluated for sickling by counting 300 cells which were classified as normal (biconcave discs) or deformed.

Minimum hemoglobin concentration required for gelation on deoxygenation was determined as previously described (2) on solutions of Hb S in 0.15 M K phosphate buffer, pH 7.35, after reaction with various amounts of HN_2 for two hours at 25°C. The proportion of methemoglobin in samples was determined spectrophotometrically before and after gelling experiments.

Solutions of hemoglobin were reacted with HN_2 as described above, but were dialyzed against 0.15 M K phosphate at pH 7.0 prior to determination of oxygen equilibria. These were performed at 25°C using tonometers with integral optical cuvettes and a Cary model 14 recording spectrophotometer (6).

RESULTS

Incubation of red cells from patients with sickle cell anemia with increasing amounts of HN_2 results in progressive inhibition of sickling (Fig. 1). Using whole blood, a concentration of at least 0.4 mg HN_2 per ml of blood is required to inhibit

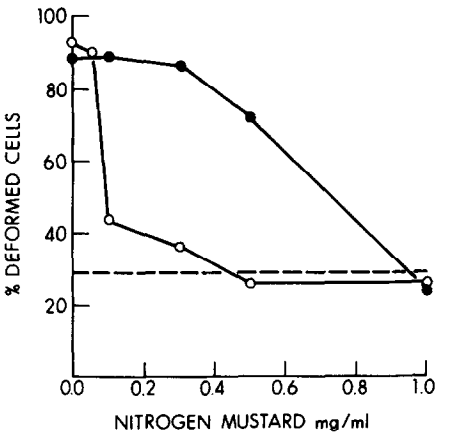
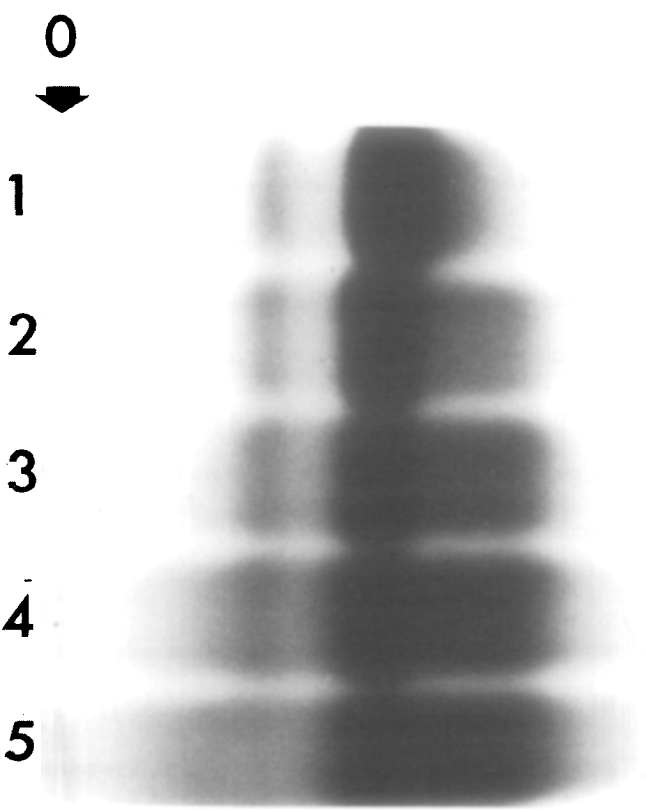


Figure 1: The effect of nitrogen mustard on erythrocyte sickling in 1% sodium metabisulfite in vitro. Solid circles: sickling in whole blood. Empty circles: sickling of RBC's in buffered saline. Dashed line: level of deformed cells before deoxygenation.



sickling; however with washed cells, inhibition is seen with as little as 0.1 mg HN_2 per ml of cell suspension. Studies with ^{14}C-labelled nitrogen mustard have shown that this agent is capable of binding to globin in intact erythrocytes. Approximately 33% of the applied amount is recovered on the isolated globin portion.

Solutions of Hb S incubated with HN_2 show electrophoretic heterogeneity which increases with the concentration of mustard (Fig. 2). There are new electrophoretic bands both cathodal and anodal to the position of Hb S with some diffuse migration of hemoglobin between these bands. Nevertheless, even with a HN_2:heme ratio of 20:1, the majority of hemoglobin migrates in the usual "S" position.

Reaction of solutions of Hb S with increasing quantities of HN_2 results in a progressive rise in the minimum concentration of hemoglobin required for gelling on deoxygenation (Fig. 3). The minimum gelling point is elevated at a mustard:heme molar ratio as low as 1:1, while at ratios greater than 5:1, gelling is no longer demonstrable. Methemoglobin content of samples reoxygenated after gelling experiments were always less than 10 per cent of the total hemoglobin, and within $\pm$ 5 per cent of the control (no HN_2) sample.

Measurements of oxygen equilibria of Hb S after reaction at a HN_2:heme molar ratio of 1:1 demonstrate normal oxygen affinity and heme-heme interaction (Fig. 4, p50=12.5 mm Hg and n value on Hill plot = 2.8). However, with a HN_2:heme ratio of 10:1, there is a small increase in oxygen affinity (p50=10 mm Hg) and a biphasic curve with two slopes; one component has an n value reduced to 1.4, while the other is near normal (n=2.37).

Figure 2: Electrophoresis on starch gel pH 8.6 of nitrogen mustard (HN_2-treated hemoglobin S hemolysates. The origin is at the left, marked O. The anode is at the right. 1) Control:Hb A_2 and Hb S. 2) Same hemolysate as 1 treated with HN_2:heme 1:1. 3) Same as 2 but HN_2:heme 5:1. 4) Same as 2 but HN_2:heme 10:1. 5) Same as 2 but HN_2:heme 20:1.

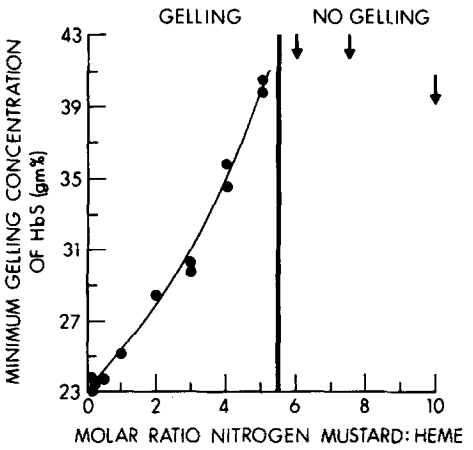


Fig. 3.

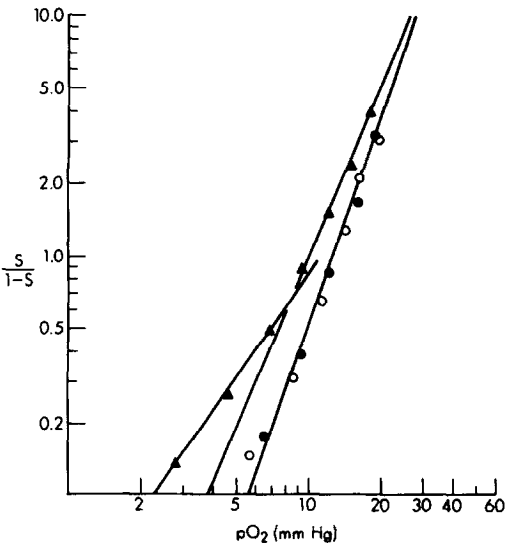


Fig. 4.

Figure 3: Minimum gelling concentrations of nitrogen mustard treated hemoglobin S. Solid circles: minimum gelling concentrations. Arrows: concentration at which experiment was stopped without gel formation.

Figure 4: Log-log plot of oxygen equilibria of hemoglobin S hemolysate (Hill plot). Ordinate: ratio of O_2 saturated hemoglobin S to unsaturated portion 1 - S. Abscissa:pO_2 in mm. Hg. Open circles: native hemoglobin S n=2.87, p50 = 12.5 mm. Hg. Solid circles: nitrogen mustard-treated hemoglobin S at molar ratio HN_2:heme 1:1. n=2.87 p50=12.5 mm. Hg. Triangles: HN_2:heme 10:1. n value for first phase = 1.4. n value for second phase = 2.37. p50 =10 mm. Hg. Temperature 25°C., pH 7.00 potassium phosphate buffer 0.15 M.

DISCUSSION

Previous studies on the reactions of nitrogen mustards with proteins have shown that the rapidly formed mustard ethylene immonium ion reacts readily with several anionic sites on proteins (7). At neutral pH, alkylation occurs most readily at the carboxyl groups of the C terminal and of the side chains of aspartate and glutamate residues, and at the ring nitrogens of histidine. (8). Esterification by alkylation of the carboxyl groups would be expected to reduce the net negative charge of the protein molecule and produce electrophoretic species with slower migration towards the anode than the native protein.

However, conformational changes produced by alkylation might expose new negative charges at the surface and thereby accelerate migration towards the anode. At low molar ratios of nitrogen mustard to heme, faster moving species predominate, while at higher molar ratios, both slow and fast species are observed. However, even the largest amount of HN_2 used in these experiments leaves most of the hemoglobin S unmodified electrophoretically; the extent of reaction of the hemoglobin with various quantities of HN_2 is not yet clear and the correlation between alkylation and change in electrophoretic mobility remains to be determined.

Alkylation of Hb S by nitrogen mustard appears to have a marked inhibitory effect on gelation of the hemoglobin solutions and in sickling of the intact red cells. Elevation of the minimum gelling concentration of Hb S above 33 gm per 100 ml, the concentration of hemoglobin within red cells, correlates with our findings that sickling of intact erythrocytes can be completely inhibited by reaction with sufficient quantities of HN_2 even when deoxygenation is nearly complete. However, much smaller elevations of the minimum gelling point may suffice to reduce the sickling tendency to a level comparable to that exhibited by red cells from persons with sickle cell trait. Since nitrogen mustard also reacts with plasma proteins (and possibly with other constituents of whole blood) it is not surprising that larger amounts of HN_2 are required to inhibit sickling in whole blood than in suspensions of washed red cells.

It is noteworthy that the amounts of HN_2 required to inhibit gelling and sickling produce neither significant methemoglobin formation in hemolysates nor an appreciable increase in the oxygen affinity. Thus gelation and sickling must be inhibited by another effect on the Hb S molecules.

It is important to consider the effects on intact red cells of treatment with large amounts of nitrogen mustard. Preliminary studies show that treatment with 1 mg HN_2 per ml red cell (whole blood) suspension produce no increase in osmotic fragility or auto-

hemolysis. The effect of mustard on enzyme-deficient red cells has not yet been examined. Fourteen red cell blood group antigens were qualitatively unchanged after exposure to HN_2, and others have observed that bank blood retains its storage properties after treatment with comparable amounts of HN_2 (9). However, "in vivo" red cell survival data would be required to determine the integrity of the treated cells.

The present data suggest that alkylation of Hb S by nitrogen mustard inhibit gelling and sickling by interfering with the polymerization of the deoxyhemoglobin, with little change in properties of the oxygen equilibrium. The very high concentrations of mustard required to inhibit sickling preclude its direct use in patients with sickle cell anemia, but the possibility of extra-corporeal treatment or the use of less toxic alkylating agents are being actively explored in our laboratory.

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REFERENCES

1. Ingram, V., Nature. 180, 326, 1957.
2. Bookchin, R.M. and R.L. Nagel, J. Mol. Biol. 60, 263, 1971.
3. Bookchin, R.M. and R.L. Nagel, Clin. Res. 19, 411, 1971.
4. Drabkin, D.L., J. Biol. Chem. 164, 703, 1946.
5. Smithies, O. Biochem. J. 71, 585, 1946.
6. Nagel, R.L., Q.H. Gibson and T. Jenkins, J. Mol. Biol. 58, 643, 1971.
7. Ross, W.C.J., in Biological Alkylating Agents, Butterworths, London, 1962.
8. Burnop, V.C.E., G.E. Francis, D.E. Richards and A. Wormall, Biochem. J., 66, 504, 1957.
9. Hartman, F.W., G.H. Mangun, N. Feeley and E. Jackson, Proc. Soc. Exp. Biol. and Med., 70, 248, 1949.