Metabolic Effects of Antisickling Amounts of Nitrogen and Nor-Nitrogen Mustard on Rabbit and Human Erythrocytes

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Nitrogen mustard (HN₂) and Nor-nitrogen mustard (Nor-HN₂) both inhibit the polymerization of deoxyhemoglobin S in solution and in intact erythrocytes. Metabolic studies were undertaken to determine the feasibility of an extracorporeal treatment with these or related agents. Glucose utilization, hexose monophosphate shunt activity, methemoglobin reduction, and incubation with acetylphenylhydrazine for Heinz body formation were performed, as well as specific assays for hexokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase, glutathione reductase, ATP, reduced glutathione (GSH), and survival of autologous mustard-treated cells in rabbits. HN₂ was found to enter red cells rapidly and bind to intracellular contents. Metabolic studies revealed no significant inhibition or alteration of function by Nor-HN₂ at 10 mg/ml of whole blood. Rabbit red cell survival was also normal. HN₂, however, inhibited glutathione reductase and blocked the free sulfhydryl group of GSH by forming several addition products of alkylated GSH. Heinz body test with acetylphenylhydrazine became positive in HN₂-treated cells, and rabbit red cell survival was shortened considerably in the concentration range used to inhibit sickling. Ascorbic acid stimulation of the hexose shunt pathway was inhibited by HN₂, but methylene blue stimulation remained unaffected. ¹⁴C-HN₂ remains bound to red cells in vivo, and the disappearance of radioactivity is similar to that found with ¹⁴C-DFP (diisopropylfluorophosphate). Oxygen affinity of both HN₂ and Nor-HN₂ treated human red cells remains virtually the same as that found in control samples. It is concluded that Nor-HN₂ may be a suitable agent for an extracorporeal therapy, and that each mustard needs to be evaluated individually for its antisickling effects and its suitability for extracorporeal use.

W E HAVE PREVIOUSLY REPORTED that nitrogen mustard (HN₂) and nor-nitrogen mustard (Nor-HN₂) both inhibit gelation of solutions of deoxyhemoglobin S and sickling in intact red cells.¹² We also reported that osmotic fragility and red cell surface antigens appeared unaltered by treatment with high concentrations of mustard. Because an effective antisickling agent must also preserve red cell viability, we have investigated some metabolic aspects of mustards which are now reported in this communication.

MATERIALS AND METHODS

Techniques

Heparinized blood was obtained from normal volunteers, patients with abnormal hemoglobins, and black males with known glucose-6-phosphate dehydrogenase (G6PD) deficiency. Hexokinase,
pyruvate kinase, G6PD, and glutathione reductase (GSSG reductase) activities, as well as reduced glutathione content (GSH), were all determined by the methods of Beutler. Adenosine triphosphate (ATP) content was determined with a Calbiochem Statpak. Methemoglobin reduction, glucose utilization, lactate and pyruvate production were all determined by previously described methods. The activity of the hexose monophosphate shunt in normal human red cells was assayed by the method of Yunis and Yasmineh, using $^{14}$C-l-glucose. Incubations were carried out for 2 hr at 37°C in 10-ml Ehrlenmeyer flasks with center wells (Metaloglass Co., Boston, Mass.) After the 2-hr incubation period, 0.5 ml Hyamine was added to the center wells, and the incubations were terminated by the addition of 1.0 ml 10% trichloroacetic acid. The vials were shaken for 1 hr more in order to trap all $^{14}$CO$_2$. Parallel chemical glucose determinations were performed, using a Boehringer Assay Kit. Results were calculated as micromoles of glucose metabolized per hour per 100 ml RBCs, and the percentage metabolized via the shunt was calculated.

Incubation of red cells with acetylphenylhydrazine (APH) for Heinz body formation was performed according to Dacie. Electrophoresis of GSH and oxidized glutathione (GSSG) and their alkylated derivatives was performed on Whatman 3MM paper, using 1.25% pyridine-acetic acid mixture at pH 4.7. Electrophoresis was carried out for 1 hr at 2800 volts on a Savant high voltage electrophoresis apparatus. The papers were stained with either 0.2% ninhydrin in acetone or with nitroprusside-cyanide stain which is specific for both SH groups and -S-S— bonds. In order to measure red cell uptake of HN$_2$ and cyanate (CNO), washed, human red cells suspended in a NaHPO$_4$ buffered isotonic saline solution, pH 7.4, were used at a packed cell volume of 40%. $^{14}$C-HN$_2$ (Sp. Act. 0.385mCi/mmmole) at a final concentration of 0.5 mg/ml (26 mM) was incubated with an aliquot of red cells at 37°C. This yielded a molar ratio of reactants (HN$_2$;heme) of 3.2:1. At the indicated times, small aliquots were removed from the incubation mixture, rapidly washed five times in cold buffered saline, and the red cells lysed by the method of Drabkin. The stroma were discarded and not further analyzed. Hemoglobin concentrations were determined by the cyanmethemoglobin method, and 0.2-ml aliquots were prepared for liquid scintillation counting by the addition of protosol-isopropyl alcohol (1:1) and 30% H$_2$O$_2$. After bleaching for 45 min, Aquasol was added, and the samples were counted on a Packard Tri-Carb Scintillation Spectrometer. Quench correction was performed with an internal standard. A similar experiment was performed using K $^{14}$CNO (0.02mCi/mmmole) at a final concentration of 25 mmoles. Oxygen equilibria on red cells suspended in NaHPO$_4$ buffered isotonic saline, pH 7.4 were performed on an IL blood gas apparatus at a packed cell volume of 40%. When needed, correction to pH 7.4 was performed with the Severinghaus nomogram. In vivo survival of mustard-treated cells was determined in white, New Zealand rabbits using $^{14}$C-diisopropyl-fluorophosphate (DFP) as a label. Twenty milliliters of heparinized rabbit blood were drawn from a marginal ear vein. The blood was mixed with an equal volume of 0.15 M NaPO$_4$ buffer, pH 7.35 with and without HN$_2$. In the case of Nor-HN$_2$, this material was dissolved in 0.11 N NaOH at an initial concentration of 20 mg/ml. By adding equal parts of Nor-HN$_2$ solution to blood, a final concentration of 10 mg/ml was achieved. After standing at room temperature for 1 hr, the cells were washed twice in buffered saline. Four microcuries of $^{14}$C-DFP were added at room temperature, and the cells were allowed to stand for 1 hr more. After a single further wash, the cells were resuspended in 20 ml of buffered saline and reinfused. Serial bleedings were performed from the marginal ear vein of the ear not used for reinjection. The washed, packed cells were prepared for liquid scintillation counting by "wet oxidation" at 75°C with 70% perchloric acid and 30% H$_2$O$_2$. Samples were counted on a Packard Tri Carb Spectrometer, and quench correction was performed with an internal standard. Results were expressed as DPM per gram hemoglobin. These results were normalized by taking day 0 as 100%. In a separate experiment, the red cells were handled in the same manner, except that instead of DFP, 10 μCi of $^{14}$C-HN$_2$ (100 μCi/2.62 mg) were used.

**Materials**

Nitrogen mustard and GSSG were obtained from the Sigma Chem. Corp., St. Louis, Mo. $^{14}$C-HN$_2$, with the label in the chloroethyl group, was obtained from the Mallinckrodt Co., St. Louis, Mo. A thin layer chromatogram of the $^{14}$C-HN$_2$ supplied by the manufacturer which showed a single peak of radioactivity was consistent with the claim of 98% purity for this material. $^{14}$C-DFP, K $^{14}$CNO, and $^{14}$C-l-glucose were all obtained from the New England Nuclear Corp., Boston, Mass.
MINUTES

NITROGEN AND NOR-NITROGEN MUSTARD

RESULTS

Initial experiments were concerned with the uptake of $^{14}$C-HN$_2$ by human erythrocytes. The uptake of K$^{14}$CNO was performed for comparison. Figure 1 demonstrates that virtually all the hemoglobin-bound HN$_2$ entered the cell and was bound within 10 min at 37°C. In contrast, cyanate continued to bind slowly over the 2-hr observation period of this experiment. After dialysis of the hemoglobin against a 0.15 M KPO$_4$ buffer, pH 7.35 for 24 hr, about one-third of the HN$_2$ was lost, but the cyanate bound to hemoglobin remained stable. This loss of mustard from dialyzed hemoglobin is characteristic of the mustard–protein interaction. The labile bonds are thought to represent reversible ester formation.$^{10}$

Our previous studies had indicated that inhibition of gelling of deoxyhemoglobin S did not occur if the alkylation pH was kept below 7.0.$^{11}$ It was therefore of great concern to monitor the pH changes of the blood which occurred during the hydrolysis of Nor-HN$_2$. At a final concentration of Nor-HN$_2$ of 10 mg/ml (56 mM), there is a theoretical release of 168 mM HCl. It was considered that a normality of 0.11 for NaOH was most suitable, because while the initial pH was about 7.7, the final pH after 1 hr was just over 7.0. Dissolving Nor-HN$_2$ in distilled water produced pH's near 6, which are well below the pK of the protein functional groups thought to be involved in the antiskickling effect of mustards. Because HN$_2$ was studied in much smaller concentrations of 1–2 mg/ml, NaOH was not required, and this material was therefore routinely dissolved in 0.15 M KPO$_4$ or NaPO$_4$ buffers.

Glucose Metabolism

Because mustards in large concentrations are capable of reacting widely with a variety of protein functional groups, it was of interest to observe their effect on the metabolism of glucose in red cells after treatment with large amounts of HN$_2$ or Nor-HN$_2$. In addition to studying glucose metabolism in red cells containing oxyhemoglobin, glucose utilization was also observed during a 22-hr period in which nitrite-induced methemoglobin was being reduced. It can be seen that in the absence of glucose, virtually no methemoglobin is reduced and that this process, therefore, is an energy requiring one (Fig. 2). There are small differences between the control and the mustard-treated samples that were noted repeatedly, but overall, the amount of methemoglobin reduced is quite
similar. During this 22-hr period, there were similar small differences in the utilization of glucose and the elaboration of lactate and pyruvate.

Hexose monophosphate shunt activity was also quite similar in mustard treated and control cells. Figure 3 shows that unstimulated red cells metabolize about 5%-10% of their glucose via the shunt in both mustard-treated and control cells. Stimulation with methylene blue increases shunt activity about six-fold in both control and mustard treated cells. However, the stimulation seen normally with ascorbate is almost totally lacking in the HN$_2$-treated cells, but not in the Nor-HN$_2$-treated ones. It is thought that methylene blue stimulates by directly oxidizing reduced NADPH Diaphorase and that ascorbate stimulates via the glutathione peroxidase-reductase cycle.$^{12,13}$ If this is indeed the case, then enzymatic effects of mustards can explain the findings of differential shunt stimulation by methylene blue and ascorbate.

There is also an effect of HN$_2$ and Nor-HN$_2$ on the red cell enzyme glutathione reductase (GSSG reductase). It can be seen that in both rabbit and human erythrocytes, HN$_2$ almost totally inhibits this enzyme at the concentrations used to inhibit sickling (1 mg/ml). Nor-HN$_2$, on the other hand, produces a relatively mild decline in the activity of this enzyme (Fig. 4). When extra FAD (flavine adenine dinucleotide) is added to the assay mixture in a preincubation step as described by Beutler,$^3$ there is no restoration of GSSG reductase activity. When one of us (E.R.) took 10 mg/day riboflavin for a week, there was a 15% rise in GSSG reductase activity, but the level after HN$_2$ treatment in vitro was similar to the results obtained prior to riboflavin administration. We
have also measured the activities of the enzymes G6PD, pyruvate kinase, hexokinase, and carbonic anhydrase. All were within 15% of normal values.

ATP content of mustard-treated cells was also studied and not found to be affected. At 2 mg, HN₂/ml, mean red cell ATP was 97.2% of control values with a range of 88%-109% in six experiments. Similarly, mean ATP content in Nor-HN₂-treated cells (10 mg/ml) was 92.6%, with a range of 80%-108% in three experiments.

In addition to an effect on GSSG reductase, an interesting effect of mustards on reduced glutathione content (GSH) itself was also noted. There is a very rapid decline in the GSH content of HN₂-treated RBCs (Fig. 5), which is not seen with five to ten times more Nor-HN₂. Using purified GSH in the same 0.15 M KPO₄ buffer, pH 7.35 at 25°, it was possible to show that GSH reacts with HN₂ at a stoichiometry of about 3:1. There was no reaction between Nor-HN₂ and the sulfhydryl group of glutathione under these conditions. Neither raising the pH to 8.35 nor reacting Nor-HN₂ and GSH in the presence of 6 M urea produced any evidence of sulfhydryl reactivity with Nor-HN₂.
Fig. 6. Electrophoretogram of GSH, oxidized GSH (GSSG), HN2-treated GSH, (molar ratio 
HN2: GSH 10:1), Nor-HN2-treated GSH (molar ratio 10:1), and HN2-treated GSSG. Anode is
to the left; pH4.7 using 1.25% pyridine acetic acid at 2800 volts for 60 min. Stain: 0.2% ninhydrin
in acetone. Arrows indicate which spots were also positive with the cyanide-nitroprusside stain
for —SH groups or S—S bonds on a companion electrophoretogram.

The colorimetric assay employed does not discriminate between oxidation of
SH groups and addition products formed by alkylation of the —SH group.
Figure 6 indicates that addition products have been formed by the reaction of
GSH with HN2. This electrophoretogram at pH 4.7 stained with 0.2% ninhydrin
in acetone shows a series of species produced by the alkylation of GSH
by HN2. These new species have a reduced negative charge. The arrows on the
electrophoretogram indicate those spots which were positive with the cyanide-
nitroprusside stain on a companion electrophoretogram. This stain is specific
for —SH groups or S—S bonds. These new spots with decreased negative
charge are not stained by the cyanide-nitroprusside technique. The control
GSH also shows some auto-oxidation to GSSG as well.

The above results suggest that cells depleted of GSH by treatment with HN2
would tolerate oxidative stress poorly. Incubation of mustard treated red cells
with acetylphenylhydrazine for 4 hr at 37°C confirmed this suggestion. Nor-
mally, less than 40% of the cells demonstrate more than five Heinz bodies per
cell after the incubation period. HN2 at 0.5 mg/ml or more rapidly converted
the cells into the pattern found with G6PD-deficient cells in which nearly 100%
of the cells have more than five Heinz bodies. Nor-HN2, on the other hand,
produced only a modest increase of 5%–10% in the number of cells with more
than five Heinz bodies. Normal values for this experiment are less than 40%
of the cells with more than five Heinz bodies per cell after 4 hr of incubation.
HN2 rapidly converts normal cells into the G6PD deficient pattern, with nearly
100% of the cells having more than five Heinz bodies per cell. Nor-HN2, on the
Fig. 7A. Red cell survival of HN2-treated rabbit erythrocytes. Abscissa: days. Ordinate: % 14C-DFP remaining. Symbols: closed circles: control; open circles: HN2 0.7 mg/ml; triangles HN2 1 mg/ml. (B) Red cell survival of rabbit red cells treated with Nor-HN2 10 mg/ml. Symbols open circles: control; triangles: Nor-HN2-treated cells. (C) Comparison of the disappearance of 14C-DEP and 14C-HN2-treated rabbit red cells in vivo. Abscissa: days; ordinate: per cent 14C-DFP or 14C-HN2 remaining. Symbols: open circles: 14C-DFP; closed circles: 14C-HN2.
other hand, causes only a moderate increase in the number of cells with more than five Heinz bodies per cell.

These studies permit a prediction that survival of HN₂-treated cells would be impaired because of their low GSH content and loss of glutathione reductase activity. Figure 7A shows the result of a ^14^-DFP rabbit red cell survival of HN₂-treated cells at a concentration of 0.7 mg/ml (open circles), and 1 mg/ml (triangles). There is approximately a 75% decrease in red cell survival in the mustard treated cells. In contrast, Fig. 7B shows that there is virtually no difference between control and Nor-HN₂-treated cells. These results are consistent with the biochemical data already described.

It was also of interest to learn whether HN₂ is rapidly lost from treated cells or whether it remains attached to intracellular contents. Figure 7C shows the result of labeling the cells with ^14^-HN₂. In this experiment, 10 µCi of ^14^-HN₂ (less than 15 µg of HN₂/ml) produced a normal red cell survival with a decline in radioactivity in the blood which closely resembles the rate of loss of ^14^-DFP. There is a period of 0-3 days during which there is a rapid loss of label followed by a slower decline which parallels the loss of RBCs. On day 45, these cells were lysed and the stromal elements were removed. Ninety-five percent of the radioactivity was found to be associated with the intracellular contents. This suggests that during the survival study, very little label is attached to the RBC membrane.

The oxygen-carrying capacity of red cells is perhaps the most important property of these cells. Our previous studies with hemoglobin solutions have demonstrated that mustard produces no marked change in the intrinsic oxygen affinity of hemoglobin. Figure 8 shows the effect of mustard on human Hb SS red cells. The effect of cyanate is shown for comparison. It can be seen that
there is only a comparatively small left shift in the mustard-treated cells and no change in the slope of the oxygen affinity curve. CNO, on the other hand, produces both a larger increase in oxygen affinity and a lower $n$ value. It is concluded that mustard-treated cells remain capable of satisfactory oxygen transport. We have also determined that the Bohr effect is normal both in red cells treated with HN$_2$ and in purified solutions of hemoglobin treated with HN$_2$.

DISCUSSION

These studies have demonstrated a significant difference between the metabolic effects of HN$_2$ and Nor-HN$_2$ on both human and rabbit red cells. The studies in vitro demonstrated that HN$_2$ (but not Nor-HN$_2$) abolished reduced glutathione and markedly inhibited the enzyme glutathione reductase and were consistent with the rabbit RBC survival studies in vivo. These experiments showed that HN$_2$ (but not Nor-HN$_2$) had a very deleterious effect on survival at concentrations which had been found to inhibit sickling. Using $^{14}$C-HN$_2$, we were also able to demonstrate that uptake of HN$_2$ by red cells was quite rapid at 37°C, and that binding to intracellular contents—presumably hemoglobin continued for the life of the cell after an initial period of rapid loss. These binding characteristics are very similar to what was found using dialyzed and undialyzed stroma-free lysates. The labile mustard–protein bonds are reversible, alkali-labile esters. Stable bonds are more likely to be formed with imidazole and sulphydryl groups.$^{14}$

The metabolic studies presented here cover three broad interrelated areas of red cell metabolism.$^{1}$ The Embden-Meyerhof pathway which accounts for about 95% of the glucose metabolized; (2) the hexose monophosphate shunt with the GSSG-GSH reduction oxidation cycle; and (3) the enzymatic reduction of methemoglobin. All of these studies have indicated only a minimal inhibition by mustards with the exception of the specific enzymes and metabolites noted. The presence of normal ATP content confirms that most aspects of intermediary metabolism are not interrupted.

The reduction of methemoglobin by red cells is thought to occur by four possible mechanisms.$^{15}$ There is an NADH dependent reductase, direct non-enzymatic reduction by GSH and ascorbate, and an NADPH methemoglobin reductase. The first pathway appears to be the major one. It is of interest to note that removal of all GSH with HN$_2$ had no effect on the amount of methemoglobin reduced. Ablation of GSH with N-ethyl maleimide (NEM) yields similar results.$^{4}$ The NADH-dependent reductase does require an intact Embden-Meyerhof pathway which is available in HN$_2$-treated cells.

The pentose shunt pathway is required for the maintenance of reduced GSH via generation of NADPH. Stimulation of the shunt by methylene blue and ascorbate are thought to occur by different mechanisms. Methylene blue is thought to transfer electrons from NADPH to oxygen via NADPH diaphorase, while ascorbate transfers electrons via GSH and the GSSG-GSH reductase–peroxidase cycle. Here, HN$_2$ is specifically able to abolish the stimulation from ascorbate and not affect the stimulation by methylene blue. The enzyme affected in this case appears to be GSSG reductase, although the peroxidase enzyme was not studied.
The blockade of GSH which occurs with HN₂ can account for all the findings of increased Heinz body formation in the presence of APH and the shortened red cell survival in rabbits. Our data do not permit us to speculate on whether red cell survival is shortened because new GSH cannot be synthesized at all or because it cannot be regenerated fast enough to protect cells which have been totally depleted of GSH.

Finally, it is important to note that oxygen affinity and oxygen transport are not impaired by subjecting red cells to large amounts of alkylating agents which are quite toxic in another context. These data underline the different effects of mustards and suggest that an alkylating agent may be synthesized which is capable of inhibiting sickling with few, if any, harmful side effects, and that it may conceivably be used in an extracorporeal procedure for the treatment of sickle red cells.

REFERENCES

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