Evaluation of Extracorporeal Alkylation of Red Cells as a Potential Treatment for Sickle Cell Anemia

By S. Charache, R. Dreyer, I. Zimmerman, and C.-K. Hsu

Nitrogen mustard and nor-nitrogen mustard inhibit sickling, but the concentrations required would be associated with unacceptable toxicity if these agents were administered to patients. Red cells could be treated extracorporeally and infused back into donors, if the alkylating agent could be removed or inactivated, if the treatment per se did not significantly shorten red cell survival, and if viable alkylated lymphocytes could be eliminated from the treated blood. To estimate whether these conditions could be met in a clinical trial, red cells from four dogs were alkylated at 6-wk intervals. No toxic reactions were observed, although not all nor-nitrogen mustard was removed by the washing procedure. Red cell survival was shortened to about half that of control cells, using concentrations of alkylating agent which reduce sickling by 50%. Lymphocytes from treated blood could still exclude trypan blue, but could not be shown to circulate after reinfusion into donor dogs. If alkylating agents are used to treat patients' cells, inhibition of sickling may outweigh the shortening of red cell life span induced by the treatment; blood should probably be irradiated before infusion to avoid administration of alkylated and potentially mutated, but viable, lymphocytes.

If red cells from patients with sickle cell anemia are treated with alkylating agents in vitro, sickling is inhibited when the cells are subsequently deoxygenated. A possible therapeutic role for these drugs is suggested, but the high concentrations required (0.1–0.3 mg nitrogen mustard/ml red cells) would produce unacceptable toxicity if administered systemically. Extracorporeal treatment of red cells with removal of unbound drug (and its reaction products) might eliminate toxic reactions in tissues other than the blood if the agent did not elute from red cells after they are reinfused into patients.

Alkylating agents are relatively nonspecific in their effect on red cells. Nitrogen mustard (HN₂) slightly decreases methemoglobin reduction and inactivates glutathione reductase, alters the physical properties of red cells, and shortens their survival. Nor-nitrogen mustard (nor-HN₂) produces less biochemical damage, but at least as much physical alteration; the survival of rabbit red cells treated with 10 mg/ml, however, is reported to be normal.

Some shortening of survival of patients' red cells could be tolerated if that decrease in life span were outweighed by the increase which would follow inhibition of sickling. Studies with an animal model of sickle cell anemia suggested that this was the case, but the conditions of those experiments were so unusual that their clinical application is unclear. Convincing data could be ob-

From the Johns Hopkins University School of Medicine, Baltimore, Md. Submitted June 17, 1975; accepted September 11, 1975.

Supported by Contract NIH-NHLI-72-2930-8 and Grant HL02799 from the National Heart and Lung Institute, Clinical Research Center Grant 5MO-1RR3511, and gifts from several community organizations, in particular the Black History Club of the Baltimore Polytechnic Institute.

Address for reprint requests: Samuel Charache, M.D., Johns Hopkins Hospital, Baltimore, Md. 21205.

© 1976 by Grune & Stratton, Inc.
tained by measurements of the survival of treated cells in patients with sickle cell anemia, but another hazard of extracorporeal treatment would have to be delineated first.

The high concentrations of nitrogen mustard used to inhibit sickling in vitro may7 or may not8 kill lymphocytes which are present in the incubation mixture. Some lymphocytes divide very slowly, and have long life spans;9 these cells are probably resistant to the lethal effects of alkylating agents,10 but may be susceptible to their mutagenic and carcinogenic action.11 Washing of erythrocytes, a necessary part of most schemes for extracorporeal treatment, does not remove all lymphocytes from the sample.12 One reinfused, but mutated, lymphocyte, if capable of dividing, could eventually produce an illness much more devastating than sickle cell anemia.

A preliminary study of extracorporeal treatment of blood seemed necessary to answer three questions: (1) Can sufficient alkylating agent be removed from treated blood to prevent marrow depression after the blood is reinfused? (2) Is there a difference between survival of red cells treated with HN2 and nor-HN2? (3) Do lymphocytes survive the extracorporeal treatment?

MATERIALS AND METHODS

Animal Studies

Mongrel dogs which had previously been quarantined, dewormed, vaccinated against distemper and rabies, and observed for 1 mo were studied for 3-6 mo. Their blood was treated at approximately 6-wk intervals: three dogs were treated only twice, while three were treated four times. Blood (200-300 ml) was collected from the jugular vein into ACD solution, using standard plastic bags; dogs were anesthetized with Surital. In some instances, 32P-DFP (Amersham Seearle Corp., Arlington Heights, Ill.) was injected directly into the jugular vein after termination of the phlebotomy.

Plasma was removed by centrifugation, and 32P, 14C, or 3H-DFP (New England Nuclear, Boston, Mass.) was added to aliquots of the blood through a 0.2 μ Millipore filter (as were all nonsterile solutions). After incubation for 30 min, at 37°C, the alkylating agent or a buffer control was added. Initially, nitrogen mustard was added as a solution of Mustargen (Merck Sharp & Dohme, West Point, Pa.) in 0.13 M PO4 buffer pH 7.0; this solution is hypertonic, and later studies were done with pure nitrogen mustard (a gift from Merck Sharp & Dohme) dissolved in the same buffer. Nor-nitrogen mustard (Aldrich Chemical Company, Inc., Milwaukee, Wis.) was dissolved in 0.1 M NaOH to attain maximal alkylating activity;13 the pH of this solution was 7.8, and the pH of packed red cells in it was 7.6. As much as 60 mg of HN2, or 1200 mg of nor-HN2, were added to 60 ml aliquots of packed cells. Red cells were incubated with alkylating agent for 1 hr, and then washed three times with isotonic NaCl solution. Unless noted, 10 cc of a 10% solution of sodium thiosulfate (Lilly Laboratories, Indianapolis, Ind.) was added during the second wash to inactivate any residual alkylating agent.14 After washing, the red cells were reinfused into a foreleg after light anesthesia with Surital. Concentrations of alkylating agent in wash solutions were measured by the method of Williamson et al.15 Standards were prepared by careful dilution of HN2 and nor-HN2 in N,N-dimethylacetamide.

Three days a week 10-cc samples of blood were obtained for hematologic studies and measurement of radioactivity. Hematocrits and white blood cell counts were done by standard hematologic methods for the 6 wk of each study; the “window” used for electronic counting of white cells was adjusted for dog blood. Platelet and reticulocyte counts were done 3 days/wk in some, but not all, studies. Measurement of 14C and 3H were carried out as previously described.15 32P was counted by dissolving packed cells in perchloric acid, and bleaching with H2O2. A “cocktail” composed of 1 part ethylene glycol monoethyl ether and 2 parts toluene (containing 6 g PPO/liter) was used for counting, which was done with a Beckman LS-100 ambient temperature scintillation counter, after chemi- and photoluminescence had subsided. Counting accuracy was 5%-7%.
and efficiency was 35% for $^3$H, 65% for $^{14}$C, and 80% for $^{32}$P. Mean life span (MLS) was calculated from the regression of disintegrations per minute (DPM) on time, according to:

$$\text{MLS}_{\text{exponential decay}} = \frac{1}{\text{slope of regression of In DPM on time}}$$

$$\text{MLS}_{\text{linear decay}} = \frac{\text{extrapolated counts on day 0}}{\text{slope of regression of DPM on time}}$$

In almost every study, regression coefficients were higher for the linear decay model, and only those data are given in Table 1.

Lymphocytes were isolated from the blood of treated dogs after centrifugation on a Ficoll-Hypaque (Ficoll, Sigma Chemical Co., St. Louis, Mo.; Hypaque, sodium diatrizoate injection, Winthrop Laboratories, Division of Sterling Drug, Inc., New York, N.Y.) gradient. The mixture used for human lymphocytes (2.4 parts 9% Ficoll: 1 part 34% Hypaque) proved unsatisfactory for canine lymphocytes, and a 4:1 mixture was used instead. Because of the different modes of extracorporeal treatment used, it was possible to compare the survival of lymphocytes exposed to HN$_2$, nor-HN$_2$, buffer alone, or no treatment (direct intravenous injection of labeled DFP).

At the end of the study, five of the six dogs were autopsied. No gross abnormalities were found, with particular attention being paid to the lymphatic system. The sixth dog is in good health at this time.

**In Vitro Studies With Patients' Cells**

The effect of alkylating agents on sickling was measured by incubating packed red cells (hematocrit 60%) from patients with sickle cell anemia with the chosen compound for 1 hr at 37°C. The cells were then diluted in 0.1 M PO$_4$ buffer (pH 7.0, 205 mOsm/liter), 0.02 M PO$_4$ in 0.13 M NaCl (pH 7.4, 279 mOsm/liter), or the patient's plasma, completely deoxygenated with nitrogen at 37°C, and fixed in 10% formalin (4% formaldehyde) in isotonic NaCl solution and counted. The proportion of sickled cells was estimated by counting 400 erythrocytes with an oil immersion objective; deformed cells which did not have sharp points, or which had an axial ratio <3, were considered "not sickled."

### Table 1. Life Span of Alkylated Canine Red Cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (mg/ml)</th>
<th>No. of Studies</th>
<th>MLS* (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer only</td>
<td>—</td>
<td>9</td>
<td>67 (43–100)</td>
</tr>
<tr>
<td>Nitrogen mustard</td>
<td>1</td>
<td>2</td>
<td>13 (12–13)</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>3</td>
<td>41 (19–75)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>4</td>
<td>43 (32–69)</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td>Nor-nitrogen mustard</td>
<td>20</td>
<td>3</td>
<td>10 (6–15)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2</td>
<td>7 (4–9)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1</td>
<td>4 (3–6)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>36 (27–47)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>102 (39–150)</td>
</tr>
</tbody>
</table>

*Calculated based on a model of linear decay. Regression coefficients were 0.73–0.99 (mean 0.90).

The mean life span of untreated cells, labeled by direct intravenous injection of DFP, was 81 days ($N = 3$). The dogs weighed approximately 20 kg; 100 ml of blood were removed during the course of a typical survival study. Six dogs were studied a total of 18 times, but since as many as three populations of tagged cells could be studied at a time, the table represents 39 survival studies (3 untreated, 9 buffer, 12 HN$_2$, and 15 nor-HN$_2$).
Typical preparations of washed "red cells" contained 10%–50% of the white cells originally present; almost all of these were mononuclear cells. The effect of prior incubation with alkylating agents on viability of human lymphocytes was estimated by pipetting alkylated packed cells over Ficoll–Hypaque solution, centrifuging at 365 g and 25°C for 40 min, removing the white cells at the interface, and staining them with trypan blue.17

RESULTS

Toxicity

No immediate neurologic or autonomic nervous system toxicity2 was noted during or after infusion of treated cells; the animals were only lightly anesthetized at the time. The treated dogs did not lose weight and showed no clinical abnormalities, except for appearing somewhat unkempt the day after anesthesia. On one occasion, addition of thiosulfate was omitted during the washing procedure: the white cell count fell to 5900 1 day after treatment, from the usual 12,000–16,000, but it rose toward normal 2 days later, and the platelet count did not change. Residual alkylating activity was not measured in this experiment. Hematopoietic toxicity was not observed in any of the other animals.

No HN2 was detected in the last wash solutions if thiosulfate was added, but not all nor-HN2 activity was removed by "washing" under conditions similar to those which might be used in a preliminary clinical trial. Residual concentrations of nor-HN2 as high as 36 μg/ml in 75 ml of red cell suspension (2.7 mg total) did not produce toxicity.

Effect on Red Cell Survival

The number of circulating tagged red cells was decreased by the blood removed 3 times a week for the studies, and survival of these cells was artificially shortened by trauma inflicted during incubation and washing.18 The actual lifespan of the erythrocytes was probably about 110 days,19 but cells labeled by direct intravenous injection of DFP "survived" only 81 days, and those incubated with buffer and then washed "survived" only 58 days. Red cells incubated with alkylating agents were compared with the latter group (Table 1). Their life span was shortened by incubation with doses of nitrogen mustard >0.1 mg/ml, and doses of nor-nitrogen mustard >3 mg/ml. Fifty per cent inhibition of sickling was produced by approximately 0.2 mg/ml of the former compound, and 3–5 mg/ml of the latter (Fig. 1). The alkylated deoxygenated cells did not have a normal appearance (Fig. 2); a concentration of HN2 that reduced sickling by 50% also reduced survival of canine red cells by 50%. Concentrations of nor-HN2 which reduced sickling to approximately the same extent shortened survival to a greater degree.

Survival of Alkylated Lymphocytes

Lymphocytes could be isolated from alkylated blood, and they could exclude trypan blue (Table 2). Dye exclusion is a very gross criterion for cell survival, and it is quite possible that some of the "living" lymphocytes had actually been "mortal" wounded by alklylation and washing. Studies by Dr. M.-F. Tsan suggested that this was the case, for glucose 1-14C oxidation to 14CO2 by al-
Fig. 1. Inhibition of sickling by nitrogen mustards. Approximately 60% of the red cells in control tubes were considered to have sickled (see text). The data shown are for cells suspended in 0.1 M PO₄ buffer, pH 7.0; very similar data were obtained when cells were suspended in plasma, or 0.02 M PO₄·0.13 NaCl, pH 7.4.

Fig. 2. (A) Control cells suspended in patient’s plasma, deoxygenated 15 min. (B) The same, after prior incubation with HN₂, 0.2 mg/ml. (C) HN₂, 0.5 mg/ml. (D) Nor-HN₂, 5 mg/ml. Alkylated cells do not have a normal appearance.

Table 2. Effect of Nitrogen Mustard on Trypan Blue Staining of Human Lymphocytes

<table>
<thead>
<tr>
<th>Treatment (mg HN₂/ml)</th>
<th>Stained Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>0.095</td>
<td>4.3</td>
</tr>
<tr>
<td>0.18</td>
<td>18.2</td>
</tr>
<tr>
<td>0.33</td>
<td>23.3</td>
</tr>
</tbody>
</table>

Packed red cells were mixed with a freshly mixed solution of Mustargen in distilled water (20 mg/ml) and incubated for 1 hr at 37°C. They were then layered over Ficoll–Hypaque (2:4:1, see Materials and Methods) and centrifuged for 30 min at 365 g and 25°C. The lymphocyte layer was removed and stained immediately.
kylated lymphocytes was inhibited by 50% when blood was incubated with 0.18 mg/ml of HN$_2$, and inhibited by 75% by a concentration of 0.65 mg/ml. Lymphocytes from treated blood were not stimulated to divide by phytohemagglutinin when cultured in the laboratory of Dr. D. Borgoankar.

When alkylated blood was tagged with DFP and reinfused into donor dogs, radioactivity could not be detected in lymphocytes isolated 1–5 wk later. Neither could radioactivity be detected in lymphocyte samples from a patient whose blood was sampled 5 and 11 days after it had been labeled and washed (but not alkylated). Failure to detect tagged lymphocytes could not be attributed to mishaps during the labeling process, for radioactive cells could easily be isolated on Hypaque–Ficoll gradients before the blood was reinfused, and a few were detectable 8 hr after infusion.

DISCUSSION

In general, our data confirm those of Roth et al. Our studies suggest that when equivalent doses of HN$_2$ and nor-HN$_2$ are compared, the latter produces greater impairment of red cell survival in dogs. Roth and his co-workers have presented evidence to the contrary, and only studies on human red cells, in patients, can yield a satisfactory comparison of the two agents. Such studies can be carried out, for as much as 60 mg of nitrogen mustard could be added to blood and then “washed out” safely if sodium thiosulfate is added to the second wash solution. Some of the thiosulfate has been reinfused into the donor animal, but its toxicity is quite low.22

Survival of lymphocytes, after exposure to a mutagen, remains as a potential hazard of the procedure. We could not demonstrate that any of the lymphocytes from alkylated blood still circulated 5–10 days later—but neither could we demonstrate the survival of lymphocytes which had not been alkylated. Our inability to do so could have been due to removal of the tagged lymphocytes during washing of the red cells or their dilution in a large extravascular pool.22

No gross evidence of lymphoproliferative disorder was present at autopsy in animals treated over a 6-mo period, but since lymphocytes can still divide more than 1 yr after labeling,23 the rate of accumulation of mutated cells could be very slow, and resulting microscopic foci of neoplastic cells could easily be overlooked.

Mustards, alkyl ureas,24 cyanate,25–27 and dimethyladipimidate28 are among a growing list of compounds which inhibit sickling, but they are probably too toxic for systemic administration. In vitro carbamylation of sickle cells has been evaluated as a safe, albeit inconvenient and expensive, mode of therapy.29,30 Nitrogen mustard has some advantages over cyanate, for much is known of its pharmacology.11,14 It does not increase oxygen affinity;1 it is inactivated by sodium thiosulfate;4 and it is readily available in a form suitable for human use. We conclude that trials of its efficacy in prolonging red cell survival can and should be carried out in patients, but that until more efficient techniques become available for complete removal of lymphocytes, alkylated blood should be irradiated31,33 before it is reinfused. Schiffer et al.24 have shown that a single dose of 35,000–50,000 rads can shorten red cell survival. Much lower doses (1500–5000 rads) have been suggested as a means to destroy lymphocytes,32,33
but the possibility remains that red cells compromised by extracorporeal alkyla-
tion might have their survival shortened still more by irradiation.

ACKNOWLEDGMENT

Dr. E. Roth of the Albert Einstein College of Medicine made unpublished data available to us and gave much helpful advice; Dr. B. Whitten of the U. S. Army Chemical Center advised us in several aspects of the study. Dr. S. Hsu and Dr. M. Williams gave not only advice and their time but reagents also; Dr. M.-F. Tsan carried out studies of glucose oxidation by alkylated lymphocytes. Mr. Louis Ross provided much help, and the studies with lymphocytes were initiated by Ms. S. R. Month and Ms. M. I. Walters.

REFERENCES


23. Norman A, Sasaki MS, Ottoman RE,


Evaluation of extracorporeal alkylation of red cells as a potential treatment for sickle cell anemia

S Charache, R Dreyer, I Zimmerman and CK Hsu