Prolongation of Sickle Cell Survival by Dimethyl Adipimidate Is Compromised by Immune Sensitization

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DIMETHYL ADIPIMIDATE (DMA), an amino-reactive crosslinking agent, is an effective antisickling agent in vitro.1,2 DMA inhibits polymerization of HbS both by increasing oxygen affinity and by a direct effect on gelation.1,3 These effects are proportional to the extent of DMA-induced inter- and intrahemoglobin cross-linking.3,4 Initial studies employed DMA at a reaction pH of 8.4. Treated under these conditions, rat red cells survived poorly when rein fused, apparently as a consequence of unfavorable changes in red cell deformability.5 However, at lower pH (7.4), the antisickling properties of DMA were preserved, but there were no longer any unfavorable effects on rat red cell survival.5 Such considerations suggested that an evaluation of the influence of DMA, employed at pH 7.4, on the survival of sickle red cells in humans was warranted. In this paper, we describe a marked prolongation in the survival of autologous DMA-treated RBC in five subjects with sickle cell anemia. In subsequent studies in three of the same subjects, RBC survival was truncated by sensitization and immune destruction.

MATERIALS AND METHODS

Subjects

The experimental subjects were adult male patients followed by the Northern California Comprehensive Sickle Cell Center. All had a history of recurrent sickle crisis, but were well at the time of study. All had a history of transfusion, but none had developed alloantibodies or been transfused within the 4 months prior to study. The experimental protocol was approved by the Human Research Committee of the University of California-San Francisco, and informed consent was obtained from each participant.

Dimethyl adipimidate (Pierce Chemical Co.) was recrystallized from methanol with ether under sterile conditions, dried under vacuum, and stored tightly sealed in the presence of desiccant. The recrystallized compound was sterile by culture and nonpyrogenic by the limulus amoeboocyte lysate assay (Ass's of Cape Cod, Woods Hole, MA). The crystals were pure, as indicated by a capillary melting point of 208-209°C. Reactivity with free amino groups was measured utilizing the strong UV absorbance of the reaction product of DMA and hydroxylamine.7 A 17-μmol/L solution of DMA was made in fresh 50 mmol/L hydroxylamine-HCl, 36 mmol/L Borax, and 31 mmol/L KH₂PO₄, pH 7.3. The increase in absorbance at 218 nm was proportional to the concentration of active DMA. No increase in absorbance was seen with hydrolyzed DMA or the orthoester formed by use of excessive ether on recrystallization. The recrystallized compound was equal in activity to the parent compound. Activity remained stable over the duration of the study.

Modification of Red Cells With Dimethyl Adipimidate

Reaction of sickle cells with DMA and 51Cr-labeling took place within a closed system of sterile bags (Fenwal), which included a treatment bag with injection port, a saline source bag, and a waste receptacle.

Blood was drawn into ACD Formula A and injected into the treatment bag. A quantity of 100-125 μCi of 51Cr as sodium chromate (Squibb & Sons, Princeton, NJ) was added, and after mixing, the blood was incubated at 39°C for 45 minutes with gentle mixing. The reaction was stopped by the addition of 50 mg of ascorbic acid. The RBC were then washed twice in 2 vol of saline and once in isotonic phosphate buffer, pH 7.7. The cells were resuspended to a hematocrit of 20% in the same phosphate buffer and prewarmed at 37°C for 10 minutes. Sufficient 200 mmol/L DMA to give a final treatment concentration of 5 mmol/L was prepared in ice-cold 0.154 mol/L phosphate buffer (pH 6.0) and immediately injected into the treatment bag through a 0.22-μm Millipore filter. The DMA reaction was allowed to proceed at pH 7.4 for 10 minutes at 37°C with gentle mixing. The modified red cells were then washed 3 times in saline, resuspended to a Hct of 50%, and reinfused into the subject. Aliquots of treated and untreated blood were retained for in vitro studies.

Blood samples were obtained periodically for 51Cr counts until at least one-half life was observed. Counts were curve fitted and the t1/2...
estimated using a computer-assisted red cell survival analysis method.\textsuperscript{4}

**Crosslinked Hemoglobin**

Intertetrameric crosslinked hemoglobin was measured after separation from single Hb tetrarers by Sephadex G100 gel filtration in 0.05 mol/L Bis-Tris NaCl 0.1 mmol/L EDTA, pH 7.4.\textsuperscript{4} Interglobin crosslinking was measured by SDS-PAGE, utilizing amide gels.\textsuperscript{10}

**Oxygen Affinity**

Whole blood oxygen affinity was measured on the Aminco Hemocan.\textsuperscript{11} Hemoglobin oxygen affinity was measured by the method of Imai et al.,\textsuperscript{12} using a Cary 118 C spectrophotometer at 578 nm and 25°C, as described by Pennathur-Das.\textsuperscript{3} Hemoglobin was stripped of 2,3-diphosphoglycerate (2,3-DPG) by exhaustive dialysis against 0.05 mol/L Bis-Tris, 0.1 mol/L NaCl, 0.1 mmol/L EDTA, pH 7.4, followed by G-25 gel filtration.

**Assessment of Sickling by Microscopy**

Sickle forms and irreversibly sickled cells (ISC) were counted under phase or bright field microscopy after fixation in phosphate-buffered 2% glutaraldehyde (pH 7.4) by established criteria.\textsuperscript{13} At least 1,500 cells were counted for each determination. Red cells were deoxygenated by exposure to 2% metabisulfide for 2 hours prior to fixation. ISC specimens were prepared by fixation after oxygenation in room air with gentle mixing for 30–60 minutes.

**Deformability**

The deformability of whole cells was evaluated by osmotic gradient ektacytometry.\textsuperscript{14} The deformability of resealed pink ghosts was also measured in the ektacytometer, using previously described techniques.\textsuperscript{15}

**Serologic Studies**

Serologic testing was done at room temperature, after incubation at 37°C in low ionic strength saline, and in the anti-human globulin phase (Coombs phase) as described by the American Association of Blood Banks.\textsuperscript{16} DMA-treated reagent cells, unless otherwise noted, were O-negative cells treated with 5 mmol/L DMA for 10 minutes at 37°C. Cells treated with 20 mmol/L concentrations of the following imidoesters for 10 minutes at 37°C were also tested for agglutination by DMA-related antibody: methyl butyrimidate, methyl acetimidate, methyl 4-mercaptobutyrimidate, dimethyl malonimidate, dimethyl 3,3'-dithio-bispropionimidate, dimethyl suberimidate, dimethyl succinimidate and isothionyl acetimidate. Group O cells were exposed to standard concentrations of ficin, papain, trypsin, and neuraminidase as recommended by the American Association of Blood Banks before or after DMA treatment and were then tested for agglutination.

**RESULTS**

The hematologic features of the five patients with sickle cell anemia who participated in this study are shown in Table 1. All had evidence of brisk hemolytic anemia. In two, the survival of \textsuperscript{51}Cr-labeled autologous red cells was documented to be considerably shorter than normal (Table 2).

Modification in vitro by DMA considerably improved the subsequent survival of sickle cells in vivo (Table 2). In four subjects, the survival of modified cells was completely normal, while in the fifth, near-normal results were obtained. No adverse effects were noted at any time during the course of the survival studies.

In vitro measures of the effect of DMA demonstrated only modest changes in hemoglobin structure and function and in red cell deformability. DMA treatment resulted in hemoglobin crosslinking (Table 2), detectable both by gel filtration and by SDS-PAGE. Whole blood oxygen affinity was not measured in the five RBC survival study participants. However, in two other sickle cell anemia patients, whole blood oxygen affinity was 41 and 42 torr in fresh blood and fell minimally (to 38 and 39.5 torr) after treatment with 5 mmol/L DMA at pH 7.4. In survival study participants, the oxygen affinity of stripped hemoglobin increased minimally, as reflected by a fall in mean p50 from 4.22 to 3.96 torr. Although there was a modest reduction in the extent of sickling, over 50% of DMA-modified cells became sickled when fully deoxygenated. Red cell deformability, assessed by ektacytometry, was often slightly lower in DMA-modified cells than in unmodified cells. The deformability of resealed RBC ghosts, however, studied in two patients, was unchanged by DMA.

The survival of DMA-treated cells was studied a second time in 3 subjects, 3–6 months following the

**Table 1. Hematologic Data**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Hb (g/dL)</th>
<th>Reticulocytes (%)</th>
<th>ISC (%)</th>
<th>HbS (%)</th>
<th>HbF (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4</td>
<td>16</td>
<td>20</td>
<td>98.5</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>6.2</td>
<td>14.6</td>
<td>17</td>
<td>96</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>8.8</td>
<td>11</td>
<td>40</td>
<td>94.2</td>
<td>5.8</td>
</tr>
<tr>
<td>4</td>
<td>8.3</td>
<td>20</td>
<td>15</td>
<td>79</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>9.3</td>
<td>11</td>
<td>16</td>
<td>95.6</td>
<td>4.4</td>
</tr>
</tbody>
</table>

**Table 2. Effect of DMA on the Survival of Sickle Red Cells**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date</th>
<th>Percent Recovery</th>
<th>t\textsubscript{51}Cr (days)</th>
<th>Intertetrameric</th>
<th>Interglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated) survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5/17/82</td>
<td>102</td>
<td>5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4/26/82</td>
<td>79</td>
<td>8.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| DMA (5 mmol/L — first exposure) |
| 1      | 12/1/81 | 82      | 28.0            | 5.2             | 7.4         |
| 2      | 1/18/82 | 85      | 33.0            | 5.6             | 7.4         |
| 3      | 1/19/82 | 90      | 20.0            | 6.7             | 8.1         |
| 4      | 2/19/82 | 103     | 28.0            | 6.3             | 9.8         |
| 5      | 3/22/82 | 105     | 27.0            | 7.2             | 6.3         |

| DMA (5 mmol/L — second exposure) |
| 1      | 5/17/82 | 102     | 1.8             | 3.2             | 4.3         |
| 2      | 6/14/82 | 79      | 3.0             | 2.8             | 8.0         |
| 4      | 8/11/82 | 88      | 2.2             | 9.0             | 10.0        |

Normal values: sickle cell anemia—3–13 days (24); normal—25–35 days (25).

*Percent of total Hb.
initial studies (Table 2). A complete set of survival curves obtained in a representative patient is shown in Fig 1A. In all 3 subjects, after a 5–7 day period, rapid elimination of treated cells ensued (t/2 1–3 days) and antibody directed to DMA-treated cells was detectable in the serum of each subject (Fig 1B). Prior to the initiation of the second study with DMA-modified red cells, antibody could not be detected in two of the three subjects. In the third (No. 1), a weakly reactive antibody, active only in low ionic strength saline at 37°C, was detectable 3 months after the initial study. There was no clinical evidence of accelerated hemolysis of unmodified red cells in any patient.

When present, the antibody was of low titer (1:16–32) and reacted most strongly in the antiglobulin test. The strength of its reaction was not affected by exposure of reagent cells to ficin, papain, trypsin, or neuraminidase, before or after DMA treatment. Fetal red cells modified by DMA were agglutinated as strongly as similarly treated adult red cells. Attempts to absorb the antibody with unmodified red cells, DMA, or the product of the reaction of DMA with hydroxylamine were unsuccessful. In contrast, complete absorption was achieved utilizing DMA-treated cells. Antibody recovered from ether elution of DMA-treated cells displayed activity similar to that of DMA antibody from serum. The antibody did not react with the subject’s own untreated red cells or with cells of a red cell antigen panel. It agglutinated red cells pretreated with any of the nine imidoesters evaluated.

**DIscussion**

Although many potential antisickling agents have been evaluated in vitro, only seven, including DMA, have been subjected to red cell survival studies in sickle cell patients (Table 3). Of these agents, the imidoesters and cyanate appear to be the most effective in prolonging red cell survival, whereas nitrogen mustard has a lesser, but demonstrable, effect. The remaining three agents evaluated (urea, zinc, and Cetiedil) are not effective, at least under the experimental conditions employed.

At the concentration of DMA employed in this study, only modest changes are seen in the usual in vitro measurements used to gauge inhibition of sickling. As we have previously shown, at pH 7.4 and 5 mmol/L DMA, the oxygen affinity of stripped hemoglobin increases by 12%, morphological evidence of sickling at low oxygen tension (0% and 3.3% O₂) decreases by 50%, and the solubility of deoxy-HbS increases by 14%. Minimal alterations in deformability of whole cells are seen, and ghost deformability is normal. The data we obtained in the red cells utilized for survival studies, allowing for differences in reagent delivery and incubation conditions, are consistent with these earlier observations. It seems surprising that a major improvement in red cell survival should be associated with such small changes in oxygen affinity or solubility of deoxy-hemoglobin S. Other effects of DMA may have a greater impact on cell survival. For example, the kinetics of gelation are strongly affected by DMA. The prolonged survival and excellent initial recovery of DMA-modified red cells suggest that even the survival of erythrocytes damaged in the sickle cell host prior to treatment is prolonged, possibly through a membrane effect. DMA restores normal cation balance in hereditary hydrocytosis red cells, and decreases the mean corpuscular hemoglobin concentration (MCHC) of sickle cells. Although a decrease in MCHC was not seen here, possibly due to the long incubation procedure, DMA may stabilize cation permeability and retard dehydration.

A potential limitation to the therapeutic implications of the greatly improved RBC survival associated with DMA treatment derives from the increase in oxygen affinity associated with the use of this agent. Theoretically, if this increase is sufficiently large, a small aliquot of DMA-treated sickle cells can remain fully oxygenated in the venous circulation, while neighboring untreated red cells become deoxygenated dur-
ing the normal process of gas exchange. Exclusion from deoxygenation would then favorably affect the survival of this small subset of red cells, but would have no relevance to the use of DMA to treat entire populations of red cells. In actuality, under the treatment conditions we employed, only a small increase in whole blood oxygen affinity (2–3 torr) was associated with DMA, and this would not be sufficient to prevent deoxygenation of treated sickle cells.

Reaction of DMA with the red cell membrane formed a potent immunogen that sensitized all three patients who underwent a second survival study using DMA-modified red cells. No patient had detectable DMA-specific antibody immediately (3–4 weeks) after primary exposure to treated RBC. Upon antigen challenge, however, all tested demonstrated an amnestic rise in antibody and accelerated destruction of treated RBC. The nature of the antigen(s) was not fully defined by our studies. The neoantigen appeared to be the result of a direct modification of the cell surface, as the antibody could be absorbed by DMA-treated cells but not by DMA itself or by the product of the reaction between DMA and primary amines. The antibody cross-reacted with cells treated with any of the other imidoesters evaluated, also suggesting that a modified membrane site, rather than the DMA molecule itself, was the neoantigen. DMA modification did not unmask an antigen that reacted with naturally occurring antibodies in the general population, as treated red cells were not polyagglutinable by control sera. Differences in red cell surface antigens occurring during normal development of the fetus or produced through removal of sialic acid or antigens vulnerable to proteolysis did not detectably decrease antigen formation of red cells exposed to DMA, suggesting that none of these antigens are vital to formation of the hapten.

Gabuzda and coworkers have demonstrated that repeated infusions of methylacetimidate (MAI) treated sickle cells can stimulate the formation of a similar antibody that then curtails the survival of treated red cells. In an attempt to limit the antigenicity of MAI-treated red cells, the same workers showed that exposure of membrane amino groups to pyridoxyl phosphate blocks their subsequent reaction with MAI. MAI-treated cells prepared in this way exhibit less agglutination when incubated with MAI-specific antibody, but are not protected from immune destruction in vivo. Although we did not evaluate such techniques for minimizing the antigenicity of imidoester-treated red cells, it is unlikely that they would be more effective with DMA-treated red cells than with MAI-treated cells. Thus, at present, immunogenicity would appear to limit the further consideration of DMA as a potential therapeutic agent in sickle cell disease.

Our experience suggests that favorable effects on sickle cell survival may not be fully predicted by traditional assays of $O_2$ affinity and hemoglobin S solubility. Membrane factors, such as deformability, or synergism of several factors may ultimately prove more important, if more difficult, to assess in vitro. In the absence of any in vitro test highly predictive of prolonged red cell survival, promising in vitro agents should also be evaluated in vivo if toxicity is not a limiting factor. Our study also serves as a reminder of the efficiency of the immune system. The lack of serologic evidence of antibody formation following initial DMA treatment in two of the three patients who subsequently exhibited sensitization demonstrates that compatibility shown in vitro may not predict the response to an in vivo antigen challenge. Therefore, we recommend that red cell survival studies should include more than a single exposure to treated cells in order to assess their antigenicity.

**ACKNOWLEDGMENT**

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