An Explanation for Nonimmunologic Adsorption of Proteins Onto Red Blood Cells: Schiff’s Base Reactions

By D. Jamin, J. Demers, I. Shulman, H.T. Lam, and R. Momparler

During a phase I evaluation of diglycoaldehyde (INOX), an intravenous chemotherapeutic agent used to treat children with malignancies, all of eight patients tested developed a positive direct antiglobulin test (DAT) in vivo. The DAT became positive within one to seven days after the first administration of the drug and remained positive for up to 12 days following the last dose. The indirect antiglobulin tests were negative. None of the patients showed clinical or laboratory evidence of hemolysis at the time the DAT became positive or during follow-up. Eluates made from the red cells of two of the eight patients were both negative by indirect antihuman globulin testing. In vitro studies with INOX and glutaraldehyde, both dialdehyde compounds, showed nonimmunologic adsorption of protein onto red cells, probably by the condensation of aldehyde groups of these compounds to form Schiff’s bases with amino acids of serum proteins and red cell membrane proteins. This reaction provides an explanation for the globulin detected on the red cells of patients treated with INOX.

INOX (diglycoaldehyde) is the periodate oxidation product of the purine nucleoside, inosine, in which carbons 2’ and 3’ have been oxidized to formyl groups (Fig 1A).2,3 In vitro studies have indicated that the formyl groups can bind to proteins by a Schiff’s type reaction and that this effect may be partly responsible for the antitumor properties of INOX.3,4 During a phase I evaluation of INOX, all of eight patients tested developed a positive direct antiglobulin test (DAT). The DAT became positive within one to seven days after the first administration of the drug and remained positive for up to 12 days following the last dose. The indirect antiglobulin tests were negative. The possibility that INOX might have caused a nonimmunologic adsorption of serum proteins onto the red cells of the patients via Schiff’s type reactions was investigated in a series of laboratory studies. Glutaraldehyde, a compound that forms Schiff’s type reactions with proteins, was included in these studies.

MATERIALS AND METHODS

Patients. Direct antiglobulin tests were performed on the red cells of eight children before, during, and after the intravenous infusion of INOX in doses of up to 1,500 mg/m² per day for 4 to 10 days per course. The sex, age, and diagnosis of each patient is recorded in Table I.

Materials. Routine techniques and commercially available reagents were used.6 Reagents used included anti-IgG + C3b + C3d + C4 (Immucor, Norcross, Ga), anti-IgG (BCA, Malvern, Pa), anti-C3b + C3d + C4 (Ortho Diagnostics, Raritan, N.J., anti-bovine albumin (Cappel, Cochranville, Pa), anti-human albumin (Cappel), 22% bovine albumin (BCA); 22% bovine albumin was diluted to make 6% bovine albumin), 16.5% human immune serum globulin (Armour Pharmaceutical, Kankakee, Ill.), 25% human albumin (Travenol, Glendale, Calif), and glutaraldehyde, 3000 ug/mL (Sigma, St. Louis, Mo). Inosine dialdehyde (INOX, NSC-118994) was supplied as a lyophilized powder by the National Cancer Institute.

Preparation of INOX solution. INOX was reconstituted in phosphate buffered saline (PBS), at either pH 7.0 or 7.4 to a final concentration of 132 mg/dL.

Incubation of normal red cells with INOX plus selected protein media. One milliliter of normal red cells was incubated at 37 °C for two hours with one mL of INOX, 132 mg/dL plus one mL of either normal human serum, normal human plasma, 16.5% human immune serum globulin, 25% human albumin, 6% bovine albumin, or 22% bovine albumin. Red blood cells incubated with INOX and normal saline were used as a control. Following the incubation, the red cells were washed three times with normal saline and tested for cell-bound protein by antiliglobulin and antialbumin tests using anti-IgG + C3b + C3d + C4, anti-IgG, anti-C3b + C3d + C4, antihuman albumin, and antibovine albumin.

Incubation of normal red cells with glutaraldehyde and selected protein media. One milliliter of normal red cells was incubated at 37 °C for one hour with one mL of 300 mg/dL glutaraldehyde (either pH 6.5 or pH 7.4) plus one mL of either normal human serum, normal human plasma, 16.5% human immune serum globulin, 25% human albumin, 6% bovine albumin, or 22% bovine albumin. Red blood cells incubated with glutaraldehyde and normal saline was used as a control. Following the incubation, the cells were washed three times with normal saline and tested for cell-bound protein by antiliglobulin and antialbumin tests using anti-IgG + C3b + C3d + C4, anti-IgG, anti-C3b + C3d + C4, antihuman albumin, and antibovine albumin.

Adsorption-elution studies. One milliliter of normal red cells was incubated for two hours at 37 °C with one mL of INOX plus one mL of 25% human albumin. The red cells were then washed six times with PBS (pH 7.4). The washed red cells were added to an equal amount of glycine buffer solution (pH 1.8). An eluate was obtained by spinning down this mixture and recovering the supernatant. Tris(hydroxymethyl)-aminomethane solution was used to neutralize the acidity of the eluate. The eluate pH was adjusted to 7.4 or 6.5 with 0.1NaOH or 0.1HCl.

One milliliter of the eluate was incubated with 0.1 mL of normal red cells at 37 °C for two hours. Following the incubation, the cells were washed three times with PBS, pH 7.4, and tested with antihuman albumin. Red cells incubated with human albumin alone and red blood cells incubated with INOX alone were used as controls.

Incorporation of radiolabeled INOX onto red cells. Radiolabeled 14C INOX was supplied by the Stanford Research Institute through Dr R. Engle of the National Cancer Institute. One milliliter each of red blood cells, normal plasma, and INOX-8-14C, at a concentration of 2 mg/mL (4.2 mCi/mmol, 14.2 microCi/mg) were incubated for up to 2 hours at 37 °C. At 5 minutes, 30 minutes, one hour, and two hours, 0.5 mL aliquots of the mixture were diluted with 6 mL of 0.9% NaCl, and centrifuged at 3,000 RPM for 5

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minutes. The precipitate was washed with 0.9% NaCl, centrifuged, and the supernatant discarded. The washing was repeated two times. Five mL of cold 5% trichloracetic acid were then added to the pellet; the mixture was centrifuged, and the supernatant discarded. The precipitate was washed in saline, and then suspended in 1 mL of NCS tissue solubilizer (Amersham) and incubated at 37 °C for 48 hours. A 0.2 mL aliquot of the solubilized material was placed on a GF/C glass fiber filter (2.4 cm in diameter), placed in Omnifluor Scintillation Fluid (New England Corp), and assayed for radioactivity using a Beckman 360 Scintillation counter. Red blood cells incubated with 5% dextrose and INOX were used as a control.

**Grading and scoring.** All grading of agglutination was in accordance with the technical manual of the American Association of Blood Banks.

### RESULTS

All eight patients developed a positive DAT within seven days after the first intravenous dose of INOX (Table 1). Heat prepared eluates were made from the red cells of two of the eight patients. Both of these eluates (pH approximately 7) were negative by indirect antihuman globulin testing. In patient #8 the DAT became positive following the first dose of INOX and in patient #4 remained positive for at least 12 days after the last dose of INOX. None of the patients showed clinical or laboratory evidence of hemolysis at any time; bilirubin concentration, hemoglobin concentration, and reticulocyte counts remained stable.

Table 2 summarizes the results of antiglobulin and antialbumin tests on normal red cells incubated with INOX and either normal human serum, normal human plasma, 16.5% human immune serum globulin, 25% human albumin, 6% and 22% bovine albumin without the addition of INOX were agglutinated by anti-lgG, antihuman albumin, and antibovine albumin, respectively, at dilutions of 1:2.

Table 3 summarizes the antiglobulin and antialbumin test results on normal red cells incubated at pH 7.4 with glutaraldehyde plus either normal human serum, normal human plasma, 16.5% human immune serum globulin, 25% human albumin, 6% bovine albumin, or 22% bovine albumin. The results were positive for all corresponding proteins except complement, and were stronger at pH 7.4 than at pH 7.0. Patients receiving Cephalexin.

### Table 1. Clinical Data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Diagnosis</th>
<th>Dose of INOX (mg/m²/d)</th>
<th>Direct Antiglobulin Test*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1†</td>
<td>M</td>
<td>2</td>
<td>Neuroblastoma</td>
<td>1000 mg/10 d</td>
<td>Neg 7th 3+[†] +6 days</td>
</tr>
<tr>
<td>2†</td>
<td>M</td>
<td>7</td>
<td>Wilms</td>
<td>1000 mg/5 d</td>
<td>Neg 2nd 2+[†] +2 days</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>16</td>
<td>ALL</td>
<td>1500 mg/5 d</td>
<td>Neg 2nd 2+[†] +8 days</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>16</td>
<td>ALL</td>
<td>1500 mg/5 d</td>
<td>Neg 2nd 2+[†] +12 days</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>9</td>
<td>Rhabdomyosarcoma</td>
<td>1500 mg/4 d</td>
<td>Neg 4th 1+[†] +7 days</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>14</td>
<td>Osteogenic sarcoma</td>
<td>1500 mg/5 d</td>
<td>Neg 3rd 1+[†] +2 days</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>11</td>
<td>Ewing's sarcoma</td>
<td>1500 mg/4 d</td>
<td>Neg 4th 2+[†] +11 days</td>
</tr>
<tr>
<td>8§</td>
<td>M</td>
<td>10</td>
<td>ALL</td>
<td>1500 mg/5 d</td>
<td>Neg 1st 1+[†] +8 days</td>
</tr>
</tbody>
</table>

*Direct antiglobulin tests were performed on blood samples taken before, during, and after therapy with INOX.
†Negative indirect antiglobulin test with eluates from red cells.
§Patient was receiving Cephalexin.
complement. When the cells were incubated at pH 6.5, all reactions were negative.

Eluates prepared from red cells that had been incubated with human albumin plus INOX reacted with normal red cells using antihuman albumin when the pH of the eluate was 7.4 but not when the pH was 6.5. Control studies, ie, eluates at pH 7.4 prepared from red cells that had been incubated with human albumin without the addition of glutaraldehyde were agglutinated by anti-lgG, antihuman albumin, and antibovine albumin, respectively, at dilutions of 1:2.

Fig 2 shows the uptake of radiolabeled INOX onto red blood cells.

**DISCUSSION**

Many drugs and chemicals are known to induce positive direct antiglobulin tests. Drugs such as penicillin, quinidine, alpha-methyldopa, and cephalosporins have been well documented as causing positive direct antiglobulin tests in patients via a variety of mechanisms. None of these mechanisms is identical to that which is proposed to explain how INOX causes a positive direct antiglobulin test in vivo.

The positive antiglobulin and antialbumin tests induced in these studies demonstrate that INOX and glutaraldehyde can cause the adsorption of proteins onto red cells. The fact that albumin was adsorbed onto red cells from serum, plasma, or human or bovine albumin media in the presence of INOX or glutaraldehyde suggests that the mechanism is nonimmunologic. The proposed mechanism by which INOX and glutaraldehyde cause nonimmunologic adsorption of proteins onto red cells is via the formation of Schiff’s bases.

INOX is the periodate oxidation product of the purine nucleoside, inosine, in which carbons 2' and 3' have been oxidized to formyl groups (Fig 1A). Glutaraldehyde is similar in structure to INOX in that it also contains two free formyl groups (Fig 1B). In vitro studies have demonstrated that formyl groups can bind to proteins by a Schiff’s type reaction. Glutaraldehyde has previously been described as forming Schiff’s type reactions with proteins.

Our hypothesis is that when red cells are incubated with either INOX or glutaraldehyde in an alkaline medium, one of the aldehyde groups of either INOX or glutaraldehyde forms a Schiff’s base with an amino group of the red cell membrane. The other aldehyde group is free to form a Schiff’s base with an amino group of a plasma protein. This proposed mechanism can explain the findings of a positive DAT in patients receiving intravenous INOX (pH of blood is 7.4) and the in vitro results described in this report. This hypothesis is supported by the observation that the adsorption of proteins onto red cells was pH dependent. Protein adsorption was detected when glutaraldehyde was used at a pH of 7.4 but not at a pH of 6.5. Less protein adsorption was detected when INOX was used at a pH of 7.0 than of 7.4. INOX and human albumin eluted from red blood cells could be readsorbed onto fresh red cells at a pH of 7.4 but not at a pH of 6.5.

Heat-prepared eluates from the red cells of two patients were nonreactive. A possible explanation is that the heat elution technique did not provide optimum conditions for the dissociation of INOX-immunoglobulin complexes from the patients’ red cells or for the readsorption of those complexes onto red cells reacted with the eluate.

The findings in these experiments correspond with the behavior of Schiff’s bases which are pH dependent, and easily hydrolyzed by acids. The attachment of INOX to red blood cells is corroborated by the uptake of radiolabeled INOX-8-C14 by red cells using techniques similar to those reported by Kimball et al (Fig 2).

INOX most likely acts to produce a positive DAT in vivo in a manner similar to the proposed in vitro mechanism, ie,
by the condensation of an aldehyde group with amino acids of serum proteins and red cell membrane proteins to form Schiff's bases. Although immunoglobulin can be detected on the red cells of patients who have received intravenous infusions of INOX, the sensitization of these red cells does not appear to result in clinical evidence of hemolysis. This might be explained by the data that suggest that INOX results in the covalent attachment of immunoglobulin to red cells via the formation of Schiff's bases and not due to the specific interaction between the Fab portion of immunoglobulin molecules and red blood cells. The failure of immunoglobulin molecules to bind to red cells via their Fab portion could result in the inaccessibility of the Fc portion of the immunoglobulin molecules to the Fc receptors present on macrophages, thus preventing both erythrocyte-macrophage interaction as well as hemolysis of the sensitized red cells.

INOX has not been used in phase I or II trials for five or six years and is not likely to be used widely in the future due to hepatic toxicity and relative ineffectiveness. However, these findings may be of potential practical importance since they offer a new explanation of how drugs can induce a positive direct antiglobulin test. In theory, any drug or chemical containing two or more aldehyde or acid groups capable of reacting with proteins under physiologic conditions could produce a positive direct antiglobulin test. Drugs or chemicals in this category include Evans blue, folic acid, maleic acid, glutaric acid, glyceryl trinitrate (nitroglycerin), menadiol sodium phosphate (synkavite), penicillic acid, phthalic acid, picric acid, sodium iodipamide (cholografin sodium), and sodium iodomethamate (iodoxyl). Studies of these and other agents are being planned or conducted by our group.

ACKNOWLEDGMENT

The competent support of Jane Morita and Marcia Christen in typing this manuscript, Jacque Tagliere for serological testing and analysis, and Drs Kenneth Shaw and Gerald Endahl for biochemical consultation is most appreciated.

REFERENCES

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