Rh Blood Group-Specific Antibodies in Immune Hemolytic Anemia Induced by Nomifensine

By A. Salama and C. Mueller-Eckhardt

Nomifensine (Merital, Alivil; Hoechst, Frankfurt, FRG), an antidepressant drug, may cause immune hemolytic anemia (IHA) of the so-called immune complex type that is believed to occur by means of an innocent-bystander mechanism. In this report we describe findings that are not consistent with this mechanism in a patient with nomifensine-induced IHA associated with renal failure. In vitro studies showed a transitory positive direct antiglobulin test (DAT) due to IgG, IgM, and C3 fixation. The causative antibodies were found to be (1) a drug-independent IgM antibody in the serum and eluate that reacted only with E-positive RBC, although the patient’s RBC were E-negative; (2) an IgG antibody in the serum and initial eluates that showed a stronger reaction with e-positive than with e-negative or Rh\textsubscript{neg} RBC, but only in the presence of ex vivo antigen (ie, urine containing the drug and all its metabolites); and (3) an IgM antibody in the serum and initially also on the patient’s RBC that, in the presence of ex vivo antigen as well as in the presence of known metabolites of the drug, agglutinated all RBC equally strongly, but was hemolytically more active against E-positive than E-negative cells. Within a few days of stopping the drug the hemolysis rapidly resolved without administration of prednisone. The DAT became negative with anti-IgG and anti-IgM, and the drug-independent anti-E disappeared, but both metabolite-dependent antibodies remained detectable in the patient’s serum. We conclude that the production and specificity of the causative antibodies in this case were controlled by a larger antigenic site, presumably consisting of the drug and/or its metabolites plus RBC antigens, rather than by epitopes of the drug or metabolites alone.

SEVERAL CASES of nomifensine-dependent immune hemolytic anemia (IHA) have been reported in the last 5 years.1-4 It is generally maintained that the RBC in these patients are damaged by the so-called immune-complex mechanism. In this type of reaction, drugs are believed to serve as hapten by conjugating with some soluble noncellular macromolecule, giving rise to subsequent formation of high-affinity antibodies against the drug. The antibodies could first react with the drug, and then the antibody-drug complexes could be adsorbed nonspecifically by the RBC, which act merely as “innocent bystanders.” This hypothesis is based on the assumption that immunization does not require conjugation of the drug with RBC and that the antibodies produced do not have specificity for red cell antigens.7

We have recently reported that nomifensine-dependent antibodies probably bind to target RBC via their Fab and not by their Fc portion by showing that, first, epitopes on the Fc portion of RBC-bound antibodies are still accessible to Fc-specific anti-IgG/IgM and, second, that nomifensine-dependent antibodies remain firmly attached to RBC if trace amounts of drug are added to the wash solution.4 Such findings are not consistent with the aforementioned mechanism of nonspecific adsorption of drug-antibody complexes on target RBC.

We now report a patient with nomifensine-dependent IHA who, in the course of the disease, developed various drug-induced antibodies with Rh specificity: a transient IgM pseudo-anti-E (mimicking an alloantibody), a metabolite-dependent IgG anti-e (detectable in serum and eluates), and a metabolite-dependent IgM anti-E that was hemolytic. These findings provide direct evidence that the epitopes seen by the nomifensine-induced antibodies in this case are not formed solely by the drug or its metabolites but at least in part by RBC-specific membrane antigens.

CASE REPORT

K.I., a 58-year-old female, had been treated for psychotic episodes with various drugs since 1960. Nomifensine was first given to the patient in 1982. On November 5, 1985, nomifensine was readministered at a dose of 75 mg/d. On November 19, 1985, she noticed weakness and jaundice. On the following day the patient was admitted to a local hospital for acute lumbar pain, malaise, and dark urine. At initial presentation the patient showed the typical signs and symptoms of acute intravascular hemolysis: she was somnolent and extremely pale, and hemoglobinuria and subsequent hemoglobinemia were present. Her total hemoglobin concentration was 4.3 g/dL (free hemoglobin was not measured), the lactate dehydrogenase content was 1,746 (normal, <200 IU/L), the bilirubin level was 6.1 mg/dL (of which 4.3 mg was indirect bilirubin), and haptoglobin was undetectable. Although the patient had never been transfused, the direct antiglobulin test (DAT) was found to be strongly positive, and an agglutinating anti-E was identified in the patient’s serum. On the assumption of drug-dependent IHA, nomifensine was discontinued and the patient was transfused with 4 units of compatible, E-negative packed RBC. Without further treatment, the hemolysis rapidly resolved, but transient renal insufficiency developed, requiring temporary hemodialysis. On December 4, 1985, the patient was transferred in good physical condition to a psychiatric hospital.

MATERIALS AND METHODS

Blood samples were collected from the patient on admission before any therapy and during hospitalization at times indicated in the Tables. A complete serological analysis (ABO, Rh antigens, antibody screening) was made by conventional techniques. For antibody identification the Resolve panel (Ortho Pharmaceutical Corp., Raritan, NJ) was used. Rh\textsubscript{neg} RBC were kindly provided by Professor S. Seidl (Frankfurt). The DAT was assayed exclusively from EDTA-

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anticoagulated blood by a tube method using commercial antisera (polyspecific antitoglobulin serum [Ortho], anti-IgG [Behringwerke AG, Marburg, FRG], anti-IgM and anti-IgA [Diaimed, Morat, Switzerland], and anti-C3d [Dako, Copenhagen]). Antibodies were eluted from sensitized RBC by the heat technique (ten minutes at 56 °C). The detection of drug-dependent antibodies, including hemolysis assays, was performed as recently described6 using nomifensine, its known three main metabolites (M1, M2, M3), and its ex vivo antigens (urine collected from a healthy volunteer before (U0, Sweden), at 4 °C. The immunoglobulin fractions (IgM, IgA, IgG) were concentrated to the original serum volume and examined for antibody activity against RBC in the presence and absence of the drug and its metabolites.

To characterize the immunoglobulin classes of the antibodies, a serum sample of the patient obtained on day 15 was chromatographed on a Sephacryl S 300 column (Pharmacia Fine Chemicals, Uppsala, Sweden), at 4 °C. The immunoglobulin fractions (IgM, IgA, IgG) were concentrated to the original serum volume and examined for antibody activity against RBC in the presence and absence of the drug and its metabolites.

RESULTS

Demonstration of a transitory anti-E in early serum samples. The patient's blood group was O Rh-positive (CCDee). Her serum contained an agglutinating anti-e in initial samples (Table 1), but not in specimens taken after day 6. Although this antibody had a relatively low titer, its agglutinating activity against e-positive cells did not change after washing the cells three times with large volumes of saline so that the indirect antiglobulin test (IAT) could not be performed. This antibody was drug independent since its reactivity was unaffected when the serum was dialyzed in large volumes of saline containing 0.2% bovine serum albumin (saline-BSA) or when the antibody was recovered by elution after absorption on e-positive RBC in the absence of the drug and its metabolites.

Characterization of RBC-bound antibodies in the DAT. The DAT was performed with RBC obtained from the patient before any treatment and at various times after administration of 4 units of E-negative packed RBC. The cells were washed either with saline-BSA or with saline containing ex vivo antigens (saline-US).6 As shown in Table 2, IgG, IgM, and C3d could be demonstrated on the RBC in both instances, but the reactions due to IgG and IgM were more pronounced when the cells were washed with saline-US. The strength of agglutination decreased with time (Table 2). No antibody could be demonstrated on the RBC after day 10. These findings indicated that the antibodies belonged to the IgG and IgM classes and were, at least partly, dependent on the presence of the drug and/or its metabolites.

Isolation of antibodies by elution. Antibodies were eluted from the patient's RBC after washing with saline-BSA or saline-US, respectively,6 and were then tested against Rh, rr, and Rhnull RBC in the presence or absence of ex vivo antigens (Table 3). Using day 0 eluates, drug-independent agglutination was observed only with Rh, RBC. Metabolite-dependent reactions were found in the IAT predominantly with rr RBC. With day 6 eluates, only metabolite-dependent positivity in the IAT was detectable; it was clearly stronger with rr than with Rh, and very weak with Rhnull RBC. After the patient had been hospitalized for 2 weeks, no antibody could be eluted from her red cells.

Immunoglobulin classes and hemolytic activity of antibodies in relation to drug dependency and Rh specificity. Whole serum from day 15 and immunoglobulin fractions (IgG, IgA, IgM) were tested concomitantly against Rh, R, rr, and when possible, Rhnull RBC in the presence of nomifensine, its metabolites (M1, M2, M3), and its ex vivo antigens. The results are summarized in Table 4. Two important observations were made. First, antibody activity was detectable in the presence of metabolites and/or ex vivo antigens (US), but not in the presence of nomifensine, clearly indicating metabolite-specific antibodies. Second, at least two (possibly three) different metabolite-dependent antibodies were noticed: an IgG antibody exclusively reactive with ex vivo antigens exhibiting predominantly anti-e specificity and an agglutinating IgM antibody preferentially reactive with M2 without obvious Rh specificity. However, when the hemolytic activity of serum and immunoglobulin fractions was analyzed (Tables 4 and 5), it became evident that hemolysis was confined to IgM antibodies and occurred mainly with Rh, RBC, possibly due to a third metabolite-specific antibody. The IgA fraction did not react with RBC.

DISCUSSION

The data clearly show that the hemolytic episode in the patient described here was the result of multiple nomifensine-induced antibodies. Like previous reports of nomifensine-dependent IHA,1,4 the hemolysis was acute, intravascular, and remitted promptly after discontinuation of the drug. Renal failure, often seen in this syndrome,4 was transient but required temporary hemodialysis.

From an immunologic point of view the most intriguing and as yet undescribed finding was the apparent Rh specificity of nomifensine-induced free as well as RBC-bound antibodies. This observation bears particular significance on our understanding of the underlying pathogenetic mechanism. Initially an anti-E was detectable in the serum and in eluates prepared from autologous RBC before transfusion. Its reactivity was clearly drug independent. The assumption that the antibody represented an ordinary alloantibody is very unlikely for the following reasons: the patient had until then never been transfused, the antibody disappeared quickly after withdrawal of the drug, and it could be eluted from autologous RBC although the patient had the Rh phenotype CCDee. We therefore favor the interpretation that the
reactivity of this antibody appeared to be directed against an 
E-like antigen on human RBC, a situation resembling the 
pseudo-anti-E specificity described in patients with autoim-
mune hemolytic anemia.8-10

The antigenic determinants of the other antibodies, either 
bound to RBC or free in the serum, appeared to consist of 
compound structures related both to metabolites of nomifens-
ine and RBC membranes. This phenomenon became particu-
larly obvious upon separate analysis of serum and immuno-
complex picture in that reactions were strong with all RBC in 
the presence of ex vivo antigen, but not in the presence of nomifensine or its known metabolites. This reaction pattern resembles again that seen with warm anti-e autoantibodies, which often react more strongly with e-
metabolites, and its cx vivo antigens (presumably comprising 
other Rh phenotypes. The IgG fraction was strongly 
globulin fractions in the presence of nomifensine, its main 
positive reaction pattern resembles again that seen with warm anti-c 
RBC in the presence of cx vivo antigen, but not in the 
the presence of M1 and M3, presumably reflecting cross-reactiv-
ity with M2.6 On the other hand, E-positive cells were found 
the presence of cx vivo antigen and M2, but weaker in the 
the presence of cx vivo antigen be explained? It is conceiv-
remained inexplicable because absorption and elution of 
anti-RBC irrespective of whether the IgM fraction or whole 
serum was used (data not shown).

Although the in vitro reaction of these antibodies seemed 
to be dependent solely on the presence of metabolites or the 
drug (augmented or exclusive reactivity in the presence of ex 
vivo antigen), the antibodies remained attached to RBC in 
even in the absence of the drug or its metabolites. This 
notion that the positive DAT is drug independent is sup-
ported by the fact that nomifensine is quickly metabolized in 
vivo12 and that the antibodies remained recognizable on 
patient’s RBC longer than six days after discontinuation of 
nomifensine and after transfusion of 4 units of packed RBC. 
It is therefore unlikely that residual metabolites were a 
prerequisite for the positive DAT. Indeed, a serum sample 
obtained from the patient ten hours after the last drug 
administration and a urine specimen of the following day did not 
seem to contain nomifensine or metabolites as indicated by 
their failure to be utilized as ex vivo antigens with known 
nomifensine-induced antibodies (sera 1 and 76). How, then, 
can the enhanced positivity of the DAT and of the eluates in 
the presence of ex vivo antigen be explained? It is conceiv-

### Table 3. Reactions of Eluates Prepared 
From the Patient’s Red Cells

<table>
<thead>
<tr>
<th>Patient’s RBC</th>
<th>Titer of Eluted Antibodies Tested by Agglutination/IAT* against RBC with Rh Antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R,R,R†</td>
</tr>
<tr>
<td>Day 0 saline-BSA</td>
<td>2/SA ‡</td>
</tr>
<tr>
<td>Day 6 saline-BSA</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>saline-U5</td>
</tr>
<tr>
<td>Day 10 saline-BSA</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td>saline-U5</td>
</tr>
</tbody>
</table>

Before elution the cells were washed in the presence (saline-U5) or 
absence of ex vivo antigens (saline-BSA) and tested as indicated.

* Using polyspecific antoglobulin serum.
† In the presence or absence of ex vivo antigen (US).
‡ Strong agglutination unchanged after washing.

### Table 4. Patient’s Serum and Serum Fractions Tested 
In the Presence of Nomifensine and its Metabolites

<table>
<thead>
<tr>
<th>Antibodies Present on Day 15 in</th>
<th>RBC Tested</th>
<th>N</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>US</th>
<th>UO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole serum rr</td>
<td>-/-</td>
<td>8/4</td>
<td>8/8</td>
<td>4/-</td>
<td>32/256</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>R,R,R†</td>
<td>-/-</td>
<td>8/8</td>
<td>8/32</td>
<td>4/-</td>
<td>64/128</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>IgG fraction† rr</td>
<td>-/-</td>
<td>8/8</td>
<td>8/32</td>
<td>8/4</td>
<td>64/128</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>R,R,R†</td>
<td>-/-</td>
<td>8/8</td>
<td>8/32</td>
<td>8/4</td>
<td>64/128</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>IgM fraction† rr</td>
<td>-/-</td>
<td>8/8</td>
<td>8/32</td>
<td>8/4</td>
<td>64/128</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>R,R,R†</td>
<td>-/-</td>
<td>8/8</td>
<td>8/32</td>
<td>8/4</td>
<td>64/128</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>Rh*,†</td>
<td>-/-</td>
<td>8/8</td>
<td>8/32</td>
<td>8/4</td>
<td>64/128</td>
<td>-/-</td>
<td></td>
</tr>
</tbody>
</table>

Tests were conducted on nomifensine (N); its metabolites M1, M2, 
and M3; its ex vivo antigen (US); and drug-free urine (UO, control).

* With polyvalent antoglobulin serum; fresh AB serum (complement) 
was added to each tube before washing.
† Immunoglobulin fractions were absorbed six times with A, RBC in 
order to remove anti-A, which would have reacted with group A Rh+Rbc.
‡ Hemolysis was observed.
able that these antibodies recognize variable structural sites on the RBC surface to which they attach rather loosely in the absence, but more strongly in the presence of the drug or its metabolites. Another possible explanation for the transiently positive DAT is a simultaneous coating of RBC with residual metabolites and antibodies. In either possibility antibody binding involves defined structures on the RBC surface. This is supported by the fact that a drug-related antibody reacts only with one type of blood cell, i.e., with platelets and not with RBC.\textsuperscript{13,14}

<table>
<thead>
<tr>
<th>Table 5. Hemolytic Activity of Metabolite-Dependent Antibodies</th>
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</thead>
<tbody>
<tr>
<td>Red Cell Rh Phenotype</td>
</tr>
<tr>
<td>Whole serum*</td>
</tr>
<tr>
<td>IgM fraction</td>
</tr>
<tr>
<td>IgG fraction</td>
</tr>
</tbody>
</table>

*Day 15 serum and fractions.
†Percent hemolysis in the presence of ex vivo antigen (US) and complement.

Considering all these findings, there is now suggestive evidence that target cells in patients with drug-induced immune cytopenias of the so-called immune-complex type do not act as innocent bystanders, but rather serve as “carrier” surfaces. Although conjugation of drugs and cells in vitro does not appear stable, it might still suffice to render this complex immunogenic in some patients. Hence, it is not surprising that the antibodies in such patients, like those in animals immunized with hapten-protein conjugates, will be quite heterogeneous with regard to their binding sites on the conjugates.\textsuperscript{15,16} A similar suggestion related to drug-induced immune cytopenias has recently been made by Habibi\textsuperscript{17} and is further corroborated by a number of reports\textsuperscript{18-23} that indicate the presence of receptors for certain drug-dependent antibodies on target blood cells.

ACKNOWLEDGMENT

We are indebted to Dr T. Schleiffer in Ludwigshafen, FRG, for the generous provision of serum from his patient and for clinical information; and to Ulrike Bruns for expert assistance in the preparation of the manuscript.

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