On the Mechanisms of Sensitization and Attachment of Antibodies to RBC in Drug-Induced Immune Hemolytic Anemia

By A. Salama and C. Mueller-Eckhardt

The mechanisms of sensitization and attachment of drug-dependent antibodies to RBC in drug-induced immune hemolytic anemias are largely speculative. Nomifensine has been incriminated in causing immune hemolysis in a large number of patients. The hemolysis was usually of the so-called immune complex type, less commonly of the autoimmune type, and more surprisingly, few patients had developed both types of hemolysis. To determine whether nomifensine (metabolite)-dependent antibodies (ndab) exhibit specificity for antigenic structures of RBC membranes, 30 ndab were tested against large panels of RBC with common and rare antigens. We found that only 14 out of 30 ndab were invariably reactive with all cells tested. Nine antibodies were, similar to the majority of idiopathic or drug-induced autoantibodies, not or only weakly reactive with Rh\textsubscript{ab} RBC. Three antibodies did not react with cord RBC and could be inhibited by soluble I antigen. The remaining four antibodies gave inhomogeneous reaction patterns or were even negative with selected RBC; their specificity could not be identified. On a Scatchard plot analysis of one ndab, a maximum of 173,000 drug-dependent antibodies of the IgG class can specifically bind per RBC in the presence of the drug. Although nomifensine and its metabolites do not attach tightly onto RBC, our results clearly indicate that RBC do not act as "innocent bystanders," but rather serve as a surface for (1) a loose attachment of drugs that possibly cause a subtle structural change in the cell antigens and, by this means, allow in vivo sensitization; and (2) a specific binding of the resultant antibodies. This concept would explain why these antibodies can be directed against drug-cell complexes, against cell antigens alone (autoantibodies), or against both in the same patient.

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Nomifensine, an antidepressant drug, has been recently withdrawn because of the frequent occurrence of life-threatening immune-mediated intravascular hemolysis. In almost all cases this syndrome appeared to reflect the typical clinical and serological picture of the so-called immune complex-mediated immune hemolysis. The hemolysis was usually transitory, intravascular, and often associated with renal failure. In vitro studies have shown that the drug does not bind firmly to target cells and that the causative antibodies react with RBC only in the presence of the drug\textsuperscript{14} and/or its metabolites, which leads to cell lysis by fixation of the terminal complement complexes.\textsuperscript{5,7} The occurrence of autoimmune hemolytic anemia secondary to therapy with the drug has also been noted.\textsuperscript{8,9}

A major, yet unresolved problem in this syndrome is the mechanism of sensitization and the true antigenic sites that determine the specificity of the resultant antibodies. The prevailing notion anticipates that drugs (haptens) first conjugate with some soluble noncellular macromolecule to give rise to the subsequent formation of high-affinity antibodies. These antibodies then combine with drugs to form antibody-drug complexes that may attach nonspecifically to RBC by an "innocent bystander" mechanism.\textsuperscript{10} 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 erythrocyte antigens.\textsuperscript{10} We have recently identified in one patient with nomifensine-induced immune hemolysis multiple nomifensine (metabolites)-dependent antibodies (ndab) that recognize Rhesus blood group antigens.\textsuperscript{11} In another report the presence of receptors on RBC for certain drugs or drug-antibody complexes has been suggested.\textsuperscript{12} Therefore, we have reexamined a large number of ndab in order to see whether they exhibit specificity for known blood group antigens. Our results show that 16 out of 30 antibodies do indeed have specificity that is not only controlled by the drug and/or its metabolites but also by defined RBC antigens, particularly of the Rhesus and I/i systems. These findings provide, we believe, supportive evidence that in some circumstances in vivo sensitization to drugs may be related to the formation of loose, but immunogenically effective drug-cell complexes.

MATERIALS AND METHODS

Antibodies. Serum samples of 30 patients with clinical and laboratory criteria consistent with the diagnosis of drug-induced immune hemolytic anemia (DIIA) of the so-called immune complex type were studied.\textsuperscript{4,8,9} Each serum had ndab of the IgG and/or IgM classes invariably reactive with pooled normal RBC in the presence of ex vivo antigen\textsuperscript{4,8} of the drug (urine containing the drug and its metabolites). Additionally, 22 eluates or sera containing RBC IgG autoantibodies (seven induced by nomifensine, four by a-methyldopa, one by cyanidanol [(+)-2-(3,4-dihydroxyphenyl)-3,5,7-chromatriol], and ten unselected "idiopathic" autoantibodies) were concomitantly studied.

RBC. The following RBC were used: (1) pooled RBC freshly obtained from three or four healthy blood donors of group O with all major Rhesus factors, ie, D, C, c, E, e; (2) A\textsubscript{2} Rh\textsubscript{ab} RBC, kindly provided by Professor S. Seidl (Frankfurt, FRG); (3) two commercially available panels of O RBC (Ortho Pharmaceutical Corp, Raritan, NJ); (4) a panel of RBC lacking high-frequency antigens or having rare antigens (see the legend to Table I) was kindly supplied by the Medical Research Council Blood Group Unit, London.

Drugs. The ex vivo antigen was urine of a volunteer collected 4.5 hours after the ingestion of 200 mg nomifensine. Purified nomifen-
sine was kindly provided by Hoechst Pharmaceuticals (Frankfurt, FRG).

**Serological procedures.** The reactivity of antibodies was tested as described. Serum samples were titrated against cells in the presence of urine before (control) and after drug ingestion (ex vivo antigen). If the sera were incompatible with the cells to be tested due to isoagglutinins (anti-A, B) samples were preabsorbed with pooled A, or B RBC. After incubation for two hours at 37°C, the reactions were read visually for agglutination. Thereafter the RBC were washed three times in saline and tested by the indirect antiglobulin test using polyvalent antiglobulin serum (Ortho).

**Inhibition tests.** Drug-dependent antibodies that showed reactivity against adult but not against cord RBC were retested with adult RBC in the presence of defatted and preheated human milk (ten minutes in boiling water) as described for anti-I.

**Absolute number of antibodies attached per RBC.** Plasma containing a pure nomifensine-dependent IgG antibody (no. 1, a gift from Dr B. Habibi, Paris) was incubated with 10 × 10^6 RBC (in 0.02 mL saline) in the presence of nomifensine or saline (control). In preceding experiments the amount of plasma and drug needed for maximum binding of antibodies to RBC had been determined (0.1 mL of plasma and 0.05 mL of a solution of 0.1 g/dL [wt/vol] nomifensine dissolved in 0.15 mol/L saline methanol [1:10]). After incubation for two hours at 37°C the RBC were separated by centrifugation through 1 mL of calcium-free Tyrode buffer (0.13 mol/L NaCl, 27 mmol/L KCl, 16 mmol/L NaHCO3, 0.36 mol/L Na2HPO4, 5 mmol/L MgCl2-6H2O, pH 7.2) that was supplemented with 0.2% bovine serum albumin, 1% glucose, and 0.001 g/dL nomifensine (final concentration) to maintain maximal binding of the antibodies onto the cells. The cells were resuspended in 0.1 mL of saline containing the drug, transferred to fresh tubes, and reincubated with serial dilutions of radiolabeled and chromato graphically purified polyclonal F(ab)2 antihuman IgG (Fc) antibodies (Jackson Immunoresearch Laboratories, Avondale, PA). After incubation (30 minutes at room temperature) the cells were centrifuged again through the same buffer, and the radioactivity of the RBC pellet was measured. Centrifugation through this buffer completely eliminates unbound molecules. All experiments were run in triplicate, including the controls.

**RESULTS**

All antibodies reacted with pooled O RBC in the presence of ex vivo antigen (agglutination and/or positive indirect antiglobulin test), but in no case in the presence of urine before ingestion of the drug (negative control). Fourteen antibodies reacted with Rhnull RBC as well as with other rare or common RBC whether they were tested undiluted or diluted. In contrast, 16 antibodies showed inhomogeneous reaction patterns with the RBC tested, and the majority of them had specificity to defined RBC antigens (Table 1). Nine of these antibodies were not or only weakly reactive with Rhnull cells, and three antibodies (nos. 5, 22, 35) were negative with cord RBC. Against a panel of common RBC, some of the former antibodies, if they were available in sufficient amounts, also exhibited specificity to “simple” Rh antigens, ie, C, c, f, or to the I antigen (Table 1).

The I specificity was also confirmed by inhibition tests using soluble I antigen in human milk (Table 2). Although the remaining four antibodies (nos. 4, 17, 18, 30) seemed to recognize certain RBC antigens, their specificity could not be identified.

To study the binding characteristics of the antibodies with homogeneous reaction patterns, we analyzed the attachment of a representative antibody by Scatchard plot analysis. For this experiment, a pure IgG ndab1 was chosen. If it is assumed that one molecule of the F(ab)2 binds to one molecule of human IgG, the maximum amount of antibody binding sites was 173,000 per RBC (Fig 1). This value is, however, only an approximation since the antibodies used

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**Table 1. ndab With Specificity for RBC Antigens**

<table>
<thead>
<tr>
<th>Case*</th>
<th>Reciprocal Titer (Agglutination/AGT)</th>
<th>Commercial Panels</th>
<th>Rare Panel†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pool</td>
<td>Rhnull</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>64/512</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2/64</td>
<td>-/-</td>
<td></td>
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<tr>
<td>11</td>
<td>2/8</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>-/-</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>64/16</td>
<td>1/1</td>
<td>Strong only with C</td>
</tr>
<tr>
<td>23</td>
<td>1/16</td>
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<tr>
<td>27</td>
<td>1/32</td>
<td>1/1(w)</td>
<td></td>
</tr>
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<td>41</td>
<td>1/4</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>-/8</td>
<td>-/-</td>
<td></td>
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<tr>
<td>5</td>
<td>-/4</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>-/8</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>4/16</td>
<td>4/16</td>
<td>Negative with cord RBC</td>
</tr>
<tr>
<td>4</td>
<td>8/1(w)</td>
<td>4/1(w)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>8/1(w)</td>
<td>8/1(w)</td>
<td></td>
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<tr>
<td>18</td>
<td>16/2</td>
<td>16/2</td>
<td>Negative with cD(e)†</td>
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<tr>
<td>30</td>
<td>-/-</td>
<td>-/-</td>
<td></td>
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</tbody>
</table>

**Abbreviations:** AGT, antiglobulin test; IHRP, inhomogeneous reaction pattern; NT, not tested; (w), weak reaction.

*Indicates the number of patients in previous studies. The results with normal RBC show some discrepancies with our previous studies since another sample of ex vivo antigen and often different serum samples of individual patients had to be used.

†The following antigens were considered: K, Kn(a-); Mc(c-a-), Lac-, KEL-, U-Fya(b-), Yt(a-), D-/D-, O-, H-, C(ea-), pp, cD(e), C(a-), h(b-), Jk(a-b-), Lu(a-b-), Ge(-2,-3), Rg(-1,cS(a-), Vel-, P2; and Lw(a-b-).
were polyclonal and only one ndab was studied. The value(s) would be more precise if purified and radiolabeled F(ab)2 fragments of ndab were used. Nevertheless, the straight-line extrapolation in Fig 1 would suggest that the antibody studied was of restricted heterogeneity. When the serum was incubated with the same cells in the absence of the drug (control), no binding of antibody was seen. Washing of the antibody-coated cells in the absence of drug removed at least 70% of cell-bound antibodies.

In comparison with ndab, ten unselected idiopathic and 12 drug-induced autoantibodies were tested against the same RBC. In all groups approximately 50% of the antibodies were not reactive with Rh\textsubscript{null} cells (Table 3), thus indicating the absence of identical binding sites for all these antibodies.

**DISCUSSION**

Since the pioneer work of Landsteiner,\textsuperscript{17} it has generally been accepted that haptons, usually substances of a molecular weight of less than 1,000 daltons, can acquire immunogenicity only after coupling to a macromolecular carrier. This compound antigen, ie, hapten-protein complex, will then display a multitude of antigenic sites that may provoke the production of various antibody populations with different specificities.\textsuperscript{18,20}

![Graph](graph.png)

**Fig 1.** Absolute number of anti-IgG molecules bound per RBC preincubated with nomifensine-dependent antibody in the presence of the drug. The background radioactivity of RBC preincubated with the same serum in the absence of the drug was measured concomitantly (control). This background radioactivity (<0.01%) was subtracted from the total amount of cell-associated radioactivity for each dilution of radiolabeled antibody.
patients had developed a true autoimmune hemolytic anemia and three patients had both types of immune-mediated hemolysis. Thus, the most convincing way to address the question whether or not the RBC act as a carrier surface for the drug and its metabolites would be to demonstrate specificity of drug-dependent antibodies for defined RBC antigens. This prompted us to reinvestigate all nomifensine-induced RBC antibodies that were available in our laboratory. In fact, most antibodies studied required certain RBC antigens for full reactivity. This indicated that the target cells in this syndrome must supply a part of (in the case of drug-dependent antibodies) or even the entire area (in the case of drug-induced autoantibodies) for sensitization and for the subsequent attachment of the resultant antibodies. This is corroborated by several lines of evidence. First, the drug-dependent antibodies recognize only one cell type, i.e., RBC and not platelets. Second, binding characteristics onto the cells exactly resemble that of true RBC antibodies in that absorbed and eluted drug-dependent antibodies can be shown to rebind by their F(ab) rather than by their Fc portion. Third, the results of the Scatchard plot analysis in this study speak for specific rather than for unspecific attachment of the antibodies to the cells. In our experience, unspecific attachment of IgG antibodies to RBC cause usually weak reactions in the antiglobulin test and in radioimmunoassays. Fourth, the heterogeneity of the ndab among patients or even in the same patient (drug-dependent antibodies and/or autoantibodies) corresponds to that seen in animals sensitized with hapten-protein conjugates. Finally, some workers have, in isolated cases, reported specificity of drug-dependent antibodies.

Thus we have reason to assume that loose binding of drugs (hapten) on cell surfaces is sufficiently immunogenic for sensitization against cell-drug complexes and/or against the cells alone. The failure to recognize in vitro the causative drugs on target cells does not preclude an interaction with blood cells in vivo. Continued provision of drug into the circulation might maintain the unbound drug concentration at a level favoring such association, whereas a less favorable gradient develops in an in vitro test carried out at a single drug concentration. If this is true, it is likely that a prolonged loose attachment of drugs and/or their metabolites onto the cells can alter natural antigens in such a way that allows the production of heterogeneous antibodies.

ACKNOWLEDGMENT

We are indebted to Professor S. Seidl (Frankfurt, FRG) for supplying Rh+ RBC, to Dr B. Habibi (Paris) for generous provision of serum 1, to Dr P.A. Tippett and Dr G.L. Daniels of the Medical Research Council Blood Group Unit (London) for the opportunity to use some of their collection of RBC of rare phenotype, and to the manufacturer Hoechst Pharmaceuticals (Frankfurt, FRG) for the drug. The excellent technical assistance of Karin Kissel and the secretarial expertise of Inge Lenssen are gratefully acknowledged.

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On the mechanisms of sensitization and attachment of antibodies to RBC in drug-induced immune hemolytic anemia

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