AET-Treated Platelets: Their Usefulness for Platelet Antibody Detection and an Examination of Their Altered Sensitivity to Immune Lysis

By Philip L. Cimo and Susan A. Gerber

2-Aminoethylisothiouronium bromide (AET) increases the sensitivity of blood cells to complement-mediated immune lysis. We compared the sensitivities of untreated or AET-treated platelets to immune lysis induced by different types of platelet antibody in the $^{51}$Cr platelet lysis test. AET platelets were 8–16 times more sensitive to autoantibody and alloantibody, but 8–16 times less sensitive to drug-dependent antibody. AET-platelets bound similar amounts of alloantibody but less drug-dependent antibody, and they lysed at higher complement dilutions than did untreated platelets. AET-platelets detected 10 of 25 autoantibodies, 9 of 9 alloantibodies, and 5 of 8 drug-dependent antibodies. Untreated platelets detected 1 of 25, 6 of 9, and 7 of 8 of these respective platelet antibodies. The use of AET-platelets in the $^{51}$Cr platelet lysis test increases its sensitivity for detecting non-drug-dependent platelet antibodies. AET-platelets resemble paroxysmal nocturnal hemoglobinuria (PNH) platelets in their enhanced sensitivity to complement-mediated lysis. They differ from PNH platelets in their insensitivity to immune lysis induced by drug-dependent antibodies and, in this respect, are similar to Bernard-Soulier syndrome platelets.

The sulfhydryl agent, 2-aminoethylisothiouronium bromide (AET), is capable of enhancing the sensitivity of normal red blood cells to complement-induced immune lysis. Red blood cells exposed to AET are approximately 20 times more sensitive than unexposed cells to lysis induced by complement-fixing antibodies, and AET-treated lymphocytes are more sensitive than untreated ones to immune-mediated lymphocytotoxicity. In these respects, AET-treated cells resemble the red cells and platelets from patients with paroxysmal nocturnal hemoglobinuria (PNH). PNH platelets have been utilized as target cells in the $^{51}$Cr platelet lysis test to increase its sensitivity for detecting platelet antibody. Since the use of PNH cells is impractical for routine antibody detection, we have examined the usefulness of AET-treated platelets as target cells in the $^{51}$Cr platelet lysis test and have compared the sensitivities of these cells and normal, untreated platelets to immune lysis induced by platelet autoantibody, platelet alloantibody, and drug-dependent platelet antibody.

MATERIALS AND METHODS

Serum

Serum samples were prepared from whole blood that was clotted for 2 hr at 37°C. Serum was stored at –20°C. Prior to testing, serum samples were treated for 30 min at 56°C. Immune platelet sera were

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obtained from 3 groups of thrombocytopenic patients: (1) 25 adults with chronic idiopathic (autoimmune) thrombocytopenic purpura (ITP); (2) 8 patients with quinine- or quinidine-induced immunologic thrombocytopenia; and (3) 9 patients extensively transfused with random donor platelets who had become allosensitized as evidenced by failure to show an expected platelet count increment at 1 hr and 24 hr following platelet transfusions on at least 2 successive occasions. The last group of patients had thrombocytopenia due to bone marrow failure, and none had splenomegaly, septicemia, or disseminated intravascular coagulation. A monospecific HLA-A2 antisera and a polyspecific HLA antisera were kindly supplied by Dr. Richard H. Aster, Milwaukee Blood Center, Milwaukee, Wisc. Control nonimmune sera were obtained either from normal blood donors or from patients with nonimmune mediated thrombocytopenia due to hypersplenism, hypoplastic anemia, or disseminated intravascular coagulation. These individuals had never been pregnant or received transfusions with blood products. Serum dilutions were prepared by additions of 0.013 M phosphate-buffered 145 mM NaCl, pH 7.4 (PBS).

Serum immunoglobulin fractions were isolated by chromatography of serum on DEAE Affi-Gel Blue (BioRad Laboratories, Richmond, Calif.). A single peak containing IgG was eluted with 0.02 M K2HPO4 buffer, pH 8.0, and a second peak containing IgA and IgM was eluted with 1.4 M NaCl, 0.02 M K2HPO4 buffer, pH 7.25. Immunoglobulin identity was determined by immunodiffusion against rabbit anti-human IgG, IgA, and IgM monospecific antibodies (Meloy Laboratory, Springfield, Va.). The individual fractions were pooled and concentrated to half their original volumes by membrane ultrafiltration.

**Platelets**

Target platelets for use in assays for platelet autoantibody or drug-dependent antibody were obtained from normal group O blood donors. For studies of platelet alloantibodies, target platelets were obtained from donors whose platelets did not result in a compatible transfusion increment when infused into the allosensitized recipient. Studies with platelet alloantibodies of HLA-A2 specificity were done using platelets from an HLA-A2-positive donor. For platelet antibody testing, target platelets obtained from the same donor were either treated with AET or left untreated. In some experiments, sham-treated target platelets were utilized. AET-treated target platelets were prepared by incubating 1 part of 51Cr-labeled platelets at 100,000/cumm in PBS (see below) with 4 parts of a 2%-8% solution of AET (2-aminoethylisothiouronium bromide, Sigma Chemical Co., St. Louis, Mo.) in PBS. The AET solution was prepared immediately prior to use and adjusted to pH 8.0 with 5N NaOH. After 15 min incubation in the AET solution at 37°C, the platelets were sedimented and washed 4 times with Rossi’s buffer8 supplemented with 8 μM ethylenediaminetetraacetate (EDTA) and resuspended in PBS at their original concentration. Sham-treated platelets were prepared identically except that AET was omitted.

**51Cr Platelet Lysis Test**

Labeling of target platelets with sodium 51chromate and the 51Cr platelet lysis test was performed by the method of Aster and Enright5 with minor modifications previously described.7 Briefly, 0.02 ml of the 51Cr-labeled platelets in PBS (100,000/cumm) were incubated for 2 hr at 37°C with 0.1 ml of fresh, EDTA-anticoagulated, autologous platelet-poor plasma used as a complement source; 0.05 ml of immune or control serum; and 0.025 ml of 0.01M magnesium chloride. In studies with quinine- or quinidine-dependent platelet antibody, 0.02 ml of a 1 mg/ml solution of the appropriate drug in water was added to the above mixture. Following incubation for 2 hr at 37°C, the platelets were sedimented at 1000 g for 25 min, and the radioactivity in the supernatants and platelet button was determined. Percent spontaneous lysis (platelet lysis in tubes containing normal serum) and percent immune lysis (platelet lysis in tubes containing immune serum and corrected for spontaneous lysis) were calculated as described previously.7 A positive test for platelet antibody was present when the percent immune lysis induced by the test serum exceeded the mean percent immune lysis plus 3 standard deviations induced by 6 or more simultaneously run normal sera.

**Antibody-Binding Experiments**

Relative amounts of antibody bound from immune sera by untreated or AET-treated platelets obtained from a single donor were measured using the 51Cr platelet lysis inhibition assay9 as modified by Kunicki and Aster.8
In the presence of 12 normal sera, AET-treated platelets demonstrated higher spontaneous lysis in the $^{51}$Cr platelet lysis test than untreated platelets (untreated platelets $7.2\% \pm 1.1\%, \text{AET-treated platelets } 21.5\% \pm 4.8\%$). The degree of spontaneous lysis increased as the percent AET used to treat platelets was increased. The spontaneous lysis of platelets treated with 4% or 8% AET was considered too high to insure specificity for platelet antibody detection, and in subsequent studies, platelets treated with 2% AET were utilized unless specified otherwise. The spontaneous lysis of sham-treated platelets was identical to untreated platelets.

AET-treated platelets were more sensitive to immune lysis induced by platelet autoantibody (ITP antibody) and platelet alloantibody. Figure 1 shows the extent

**HLA Alloantibody Screening**

Some sera were screened for the presence of lymphocytotoxic antibodies by the standard National Institutes of Health microlymphocytotoxicity test\(^{11}\) against a panel of 59 HLA-typed donors (antibody testing courtesy of Dr. Robert Kermann, Tissue Typing Laboratory, University of Texas Medical School at Houston).

**RESULTS**

In the presence of 12 normal sera, AET-treated platelets demonstrated higher spontaneous lysis in the $^{51}$Cr platelet lysis test than untreated platelets (untreated platelets $7.2\% \pm 1.1\%, \text{AET-treated platelets } 21.5\% \pm 4.8\%$). The degree of spontaneous lysis increased as the percent AET used to treat platelets was increased. The spontaneous lysis of platelets treated with 4% or 8% AET was considered too high to insure specificity for platelet antibody detection, and in subsequent studies, platelets treated with 2% AET were utilized unless specified otherwise. The spontaneous lysis of sham-treated platelets was identical to untreated platelets.

AET-treated platelets were more sensitive to immune lysis induced by platelet autoantibody (ITP antibody) and platelet alloantibody. Figure 1 shows the extent
of immune lysis of untreated and AET-treated platelets induced by increasing dilutions of a serum containing ITP antibody and a serum containing polyspecific HLA platelet antibody. The AET-treated platelets showed an approximate eightfold greater sensitivity to the ITP antibody and an approximate 16-fold greater sensitivity to the HLA antibody than did untreated platelets. In contrast, the sensitivity of platelets to quinine- or quinidine-dependent platelet antibodies was lessened 8–16-fold by AET treatment (Fig. 2). When 4% AET-treated platelets were utilized as target cells, the sensitivity to the quinine-dependent antibody could be further reduced without altering the increased sensitivity to alloantibody (Table 1). The sensitivity of sham-treated platelets to ITP antibody, polyspecific HLA antibody, and a quinidine-dependent antibody was identical to untreated platelets.

One possible explanation for altered immune lysis of AET-treated platelets was altered binding of platelet antibody by these platelets. The relative amount of platelet antibody bound to untreated and AET-treated platelets was measured by comparing their capacity to inhibit immune lysis in the 51Cr platelet lysis test. Untreated and AET-treated platelets bound similar amounts of a multispecific HLA antibody (Fig. 3A), whereas AET-treated platelets bound less quinidine-dependent antibody than did untreated platelets (Fig. 3B).

To determine if the increased lysis of AET-treated platelets by non-drug-dependent platelet antibodies was due to enhanced sensitization of these cells to complement fixation, an HLA-A2 alloantibody was diluted so that it gave nearly

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**Table 1. Percent Immune Lysis of Untreated Platelets or Platelets Treated With Increasing Concentrations of AET Induced by Quinidine-Dependent Antibody and HLA-A2 Alloantibody**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Dilution</th>
<th>Untreated Platelets</th>
<th>2% AET-Treated Platelets</th>
<th>4% AET-Treated Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinidine-dependent</td>
<td>1:2</td>
<td>69</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>1:2</td>
<td>62</td>
<td>84</td>
<td>83</td>
</tr>
<tr>
<td>Normal</td>
<td>undil.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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Fig. 3. Inhibition of multispecific HLA antibody-induced (left) or quinidine-dependent antibody-induced (right) 51Cr platelet lysis by untreated (●—●) or AET-treated (○—○) platelets. Antisera were incubated for 2 hr at 37°C with equal volumes of unlabeled platelets in PBS at final concentrations indicated on the abscissa. This incubation was done in the presence of 10−6 M quinidine for the quinidine-dependent antiserum. Following incubation, complement and untreated 51Cr-labeled target platelets (autologous to unlabeled platelets) were added. After an additional 2-hr incubation, percent immune lysis, indicated on the ordinate, was determined.
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Fig. 4. Percent immune lysis of untreated [——] and AET-treated [——] platelets induced by constant dilutions of HLA-A2 antibody in the presence of decreasing amounts of complement. Antibody dilutions were chosen that gave nearly identical amounts of immune lysis of untreated and AET-treated target platelets (1:2 with untreated and 1:64 with AET-treated platelets). Complement dilutions were prepared by additions of heat-inactivated (56°C x 30 min) autologous plasma to fresh plasma used as the complement source.

identical amounts (±5%) of immune lysis with untreated or AET-treated platelets. The percent immune lysis of untreated and AET-treated target platelets was then determined in the presence of this antibody dilution with progressive dilutions of the complement source. The results of these experiments are shown in Fig. 4. Untreated platelets showed a progressive decrease in immune lysis as the complement source was diluted. However, AET-treated platelets continued to lyse at higher dilutions of complement. AET-platelets did not lyse in the absence of complement.

Sera from 42 thrombocytopenic patients with immune thrombocytopenia and 12 patients with nonimmune thrombocytopenia were assayed for platelet antibody in the ¹¹¹Cr platelet lysis test utilizing either untreated or autologous AET-treated platelets as target cells. The results are shown in Table 2. Only 1 of 25 sera obtained from patients with chronic ITP gave a positive reaction with untreated platelets (antibody titer shown in Fig. 1), whereas 10 gave positive reactions with AET-treated platelets. Of the 10 ITP patients who demonstrated positive reactions, 6 were females who had been pregnant one or more times. One, in addition, had been exposed to transfused blood products, consisting of 10 U of packed red cells and 6 U of platelet concentrate given 2 wk prior to antibody testing. The positive-reacting ITP sera were screened for lymphocytotoxic antibody, and none was detected, making it unlikely that the positive reactions were mediated by HLA

Table 2. Results of the ¹¹¹Cr Platelet Lysis in Thrombocytopenic Patients Utilizing Either Untreated or AET-Treated Platelets

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number Tested</th>
<th>Number Positive</th>
<th>Mean Percent Immune Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Untreated Platelets</td>
<td>AET-Treated Platelets</td>
</tr>
<tr>
<td>Chronic ITP</td>
<td>25</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Allosensitized</td>
<td>9</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Drug (quinine or quinidine)</td>
<td>8</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>immune thrombocytopenia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonimmune thrombocytopenia</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean for positive-reacting sera.
alloantibodies. Six of 9 alloantibody-containing sera reacted positively with untreated platelets, and all 9 gave positive reactions with AET-treated platelets. The percent immune lysis induced by the positive-reacting sera was greater with AET-treated platelets than with untreated platelets. Quinine- or quinidine-dependent antibody-containing sera, however, were less likely to be detected when AET-treated platelets were used as target cells. Seven of 8 sera reacted with untreated platelets, but only 5 reacted with AET-treated platelets, and the mean percent immune lysis induced by the positive sera was lower with AET-treated than with untreated platelets.

Immunoglobulin fractions prepared from two of the positive-reacting sera in each platelet antibody class were assayed against AET-treated platelets. Each serum fractionated showed platelet lytic activity in the IgG fraction only.

No positive reactions were encountered when sera from patients with nonimmune thrombocytopenia were tested with either untreated or AET-treated platelets (Table 2).

DISCUSSION

AET enhances the sensitivity of normal red blood cells and lymphocytes to complement-mediated immune lysis. In this study we compared the sensitivities of AET-treated and untreated platelets to lysis induced by different types of platelet antibody. AET-treated platelets were found to be more sensitive than untreated platelets to lysis induced by ITP antibody or alloantibody, but less sensitive to lysis induced by quinine- or quinidine-dependent platelet antibody.

Alterations in immune lysis of platelets exposed to AET could reflect either changes in the amounts of antibody bound to target platelets or the induction of a PNH-like defect, whereby target platelets acquire an increased sensitivity to the lytic effects of complement. Our experiments show that the enhanced lysis of AET-treated platelets by platelet alloantibody is the result of increased complement sensitivity, since AET-treated platelet, lysed at higher complement dilutions than did untreated platelets but bound similar amounts of the antibody. Increased complement sensitivity probably also accounts for the increased lysis of AET-treated platelets by ITP antibody. However, comparative measurements using ITP antibody were not made because only one ITP antibody reacted with both untreated and AET-treated platelets and it was not available in sufficient quantities for further study. Since quantitative complement binding was not measured, it is not known whether the enhanced sensitivity of AET-treated platelets to complement reflects increased binding of complement components or increased lytic effectiveness of complement or both.

The enhanced complement sensitivity of AET-treated platelets may permit their reaction with weakly complement-fixing platelet antibodies. The ITP antibody and some platelet alloantibodies give negative reactions with normal platelets in conventional complement fixation tests. Yet, some of these antibodies have been shown to possess complement-fixing properties and, thus, may be detectable if complement-sensitive AET-treated target platelets are utilized.

Quinine- and quinidine-dependent antibodies were less reactive with AET-treated platelets. This was unexpected, since it is generally believed that these antibodies form immune complexes with drugs that are strongly complement-fixing
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in reactions with platelets. AET-treated platelets bound less drug-dependent antibody than untreated platelets, and therefore, it is likely that the decrease in sensitivity of AET-treated platelets to these antibodies is accounted for by an AET-induced platelet membrane alteration that prevents the binding of drug immune complexes. In this respect, AET-treated platelets resemble the platelets of patients with the Bernard-Soulier syndrome, which show absent reactivity with drug-dependent antibodies due to deletion of the receptor (?Fc) to which these antibodies bind. An alteration of the platelet Fc receptor by AET may result in diminished binding of drug immune complexes. However, this effect alone would not explain the enhanced complement sensitivity of AET-treated platelets, since Bernard-Soulier platelets react normally to non-drug-dependent platelet antibodies. Another effect of AET, such as the glycoprotein alteration demonstrated by Righetti et al. in PNH and AET-treated red cells, might account for changes in complement sensitivity.

AET-treated platelets resemble PNH platelets in their excessive sensitivity to complement-mediated immune lysis. As is the case with PNH platelets, AET-treated platelets are useful as target cells in the 51Cr platelet lysis test. Their capacity to react with some platelet autoantibodies allows the laboratory identification of some patients with ITP. Additionally, their enhanced reactivity with platelet alloantibodies indicates that AET-treated platelets may be useful as target cells in platelet crossmatch tests. Unlike PNH platelets, however, AET-treated platelets are less likely than untreated platelets to detect quinine-, quinidine-, and possibly other drug-dependent platelet antibodies.

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