Anti-Thymocyte Globulin Induced Thrombocytopenia

JOHN LAZARCHICK, M.D.*, RENE RUSSELL, MLT. and BRENDA HORN, MT.

Department of Pathology/Laboratory Medicine
Medical University of South Carolina
Charleston, SC 29425

ABSTRACT

Anti-thymocyte globulin is strongly reactive with platelets as measured by flow cytometric analysis. This reactivity appears to be independent of human leukocyte antigen (HLA) phenotype, is dose-dependent with saturation of platelet binding sites readily achieved with concentrations of horse anti-thymocyte globulin (ATG) at 0.25 mg per ml, and cannot be blocked with human anti-PLA antibody nor with heat-aggregated human IgG. Results of immunoblot studies after electrophoresis of platelet membrane proteins are consistent with the proposal that horse ATG contains antibodies specific for platelet glycoproteins V and IIa.

Introduction

Anti-thymocyte globulin has proven to be effective in treating aplastic anemia and related disorders either alone or as an adjunct to achieve immunosuppression prior to bone marrow transplantation. One of the adverse side effects of this therapeutic agent has been the development of thrombocytopenia. The involvement of platelet glycoproteins in the development of drug-induced immune thrombocytopenia has been well documented for certain medications. This study sought to determine if the thrombocytopenia seen with anti-thymocyte globulin was on an immune basis and, if so, to determine if platelet specific antigens were involved.

Materials and Methods

FLOW CYTOMETRIC ANALYSIS

Horse anti-human platelet antibodies were detected using a modification of the flow cytometric analysis procedure previously described. In brief, 0.01 ml of target platelets was incubated with 0.1 ml of horse anti-thymocyte globulin (ATGAM)* diluted with Dulbecco’s buffered saline containing 0.05 percent sodium ethylenediamine tetraacetic acid (EDTA), 0.02 percent sodium azide and

* Address reprint requests to: John Lazarchick, M.D., Department of Pathology/Lab Medicine, 171 Ashley Avenue, Charleston, SC 29425.
one percent bovine serum albumin, pH 7.5 or with normal non-immune horse serum for 60 minutes at room temperature. The mixtures were then extensively washed, centrifuged and the platelet pellets incubated with 0.1 ml of fluorescein-conjugated goat (Fab')2 anti-horse IgG anti serum for 15 minutes at room temperature. After repeat centrifugation and washing, the platelet pellets were resuspended in phosphate-buffered saline to a final volume of two ml. The amount of fluorescein-conjugate found in the platelet suspensions was then measured using flow cytometric analysis. For this assay, region A on the spectrum III was defined as consisting of all 255 fluorescent intensity channels. The intensity of fluorescence, expressed as the mean value of region A (MCHA), is proportional to the amount of horse IgG bound to the platelet membrane. The mean channel A readings for the test serum plus platelet mixture and the control serum plus platelet mixture were determined. A ratio of relative fluorescence was then calculated. A ratio of >1.3 of test serum/control serum is compatible with the presence of platelet directed antibody in the test serum. Washed HLA-typed human pooled platelets were used as the target cells for these experiments. To assess for HLA specificity, single donor HLA-typed washed platelet preparations were used where indicated.

Experiments were also performed to determine if horse ATG binding to platelet glycoproteins could be inhibited by preincubation of the target platelets with specific anti-human platelet antibodies. The platelet antibodies were obtained using sera from a patient with idiopathic thrombocytopenic purpura (ITP), and a patient with anti-PLA1 antibody. In addition, aggregated human IgG was prepared by heating a 10 mg per ml of solution of IgG at 63°C for 20 minutes. Dilution of these IgG sources were incubated for 30 minutes at room temperature with the HLA-typed platelet preparations as described previously. The mixtures were then washed, centrifuged, and the platelet pellets reacted with dilutions of either normal horse serum or anti-thymocyte globulin and the flow cytometric analysis performed as previously described.

**Platelet Preparation/Electrophoresis/Immunoblotting**

Platelet rich plasma was prepared from blood collected in EDTA tubes by centrifugation at 150 g for 20 minutes. The supernatant was then centrifuged at 1300 g for 10 minutes. The platelet button was then resuspended in Ringer’s-EDTA containing 10 mM benzamidine HCL and 100 µg per ml of soybean trypsin inhibitor. After several washings, sufficient volume was added to achieve a platelet concentration of $1 \times 10^9$ ml. To this final suspension was then added 1:40 volume of 40 percent Triton X-100. The platelet suspension was then incubated at 37°C for one hour and refrigerated at 4°C overnight.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out as described by Laemmli using a five to 20 percent gradient gel as the separating gel and five percent polyacrylamide as the stacking gel.4 Twenty-five µl of platelet suspension ($1 \times 10^9$ platelets) were mixed with 25 µl of a solution containing 0.002 percent bromphenol blue, four percent sodium dodecyl sulfate and 0.125 M Tris, and 20 percent glycerol, pH 6.0, and incubated for five minutes at 100°C prior to loading. Electrophoresis was carried out for 16 hours at 20 volts. The voltage was increased to 80V for two additional hours.

Proteins were transferred from the polyacrylamide gel to nitrocellulose membrane was described by Towbin et al.8 The portion of the paper carrying the
molecular weight standards was stained with 0.125 percent Coomasie blue, and the remainder was cut into strips corresponding to the electrophoresis lanes and placed in blocking buffer containing five percent non-fat dry milk in 0.01 M Tris, 0.17 M sodium chloride, pH 7.5.

After an overnight incubation at room temperature, the strips were extensively washed and then incubated with appropriate antibody-containing preparations. These included mouse monoclonal anti-human GP IIIa antibody diluted 1:500 in Tris buffered saline. The normal horse serum and horse ATG were both diluted 1:500 in the same buffer. The strips were then incubated at room temperature overnight on a shaker bath. After washing, a solution of peroxidase-conjugated goat antibody to either mouse IgG or horse IgG was added to the appropriate strips and the strips incubated for one hour at room temperature. The strips were then developed using 4-chloro-1-naphthol as an indicator reagent (figure 1).

Results

The initial experiments were performed to determine if horse IgG in the anti-thymocyte globulin preparation (ATGAM) would bind to human platelets in a dose-responsive manner. For those experiments, both horse ATG and the normal horse serum were diluted to give final concentrations of IgG of approximately 0.005, 0.01, and 0.05 mg per ml prior to incubation with the HLA-typed platelet source. The intensity of fluorescence of the mean channel of A for the normal horse serum was essentially unchanged being 7.9, 7.8, and 8.1, respectively, at these concentrations. In contrast, the MCHA of the horse ATG preparation showed a progressive increase from 10.3, 12.2, to 14.2 respectively. This dose-response pattern was present irrespective of the presence or absence of human serum from controls or from the patient who had developed marked thrombocytopenia after receiving horse anti-thymocyte globulin (ATG).

Pre-incubation of the platelets with either normal human serum or normal horse serum did not inhibit subsequent binding with horse serum nor the extent of binding, i.e., similar MCHA values were obtained. The concentration of immunoglobulin from these sources was increased to 0.25 and 1.0 mg per ml. The amount of non-specific binding using the control horse serum minimally increased.

![Figure 1](image_url)
to give a MCHA value of 12.2. The MCHA value for the horse ATG dilutions increased also but plateaued with a value of 36. Further increases in the concentration of the horse ATG preparation did not show any additional increased binding suggesting that the IgG binding sites on the platelets were fluorescence histogram obtained with washed HLA-typed platelets after incubation with normal horse serum and horse ATG.

To determine whether or not this binding of IgG in the horse ATG preparation was specific for HLA antigens, similar studies were performed using single donor HLA-typed platelets from three individuals of variable haplotypes at the \( A \) and \( B \) loci. These data are shown in table I. Non-specific binding with normal horse serum was relatively constant among the three platelet sources and gave similar values to the HLA-typed platelet pool. The IgG binding with the horse ATG preparation, in contrast, was greatly increased, and the MCHA ratios were similar among the three single donor platelet sources. These results are strongly suggestive that the reactivity noted is independent of the HLA loci but since single loci were shared among this patient group, i.e., A2 in donors 1 and 2, and B60 in donors 2 and 3, it did not absolutely exclude this possibility.

Next, it was sought to determine whether or not horse ATG binding could be inhibited by pre-incubating the HLA-platelet pool source with specific anti-platelet antibodies obtained from an individual with idiopathic thrombocytopenic purpura and from a patient who had anti-Pl\(^\text{a}^\text{1}\) antibody in her serum. In addition, heat aggregated human IgG was used to block platelet Fc receptors on the premise that the IgG in the horse serum preparation was binding to these receptors. The results of these studies are shown in table II. Both sources of human anti-platelet antibody had no effect on the extent of horse ATG binding as judged by the MCHA values. Preincubation of the platelets with non-aggregated IgG had minimal inhibitory effect on horse ATG binding. The preincubation with heat aggregated human IgG did show an increased MCHA value when the platelets were subsequently incubated with normal horse serum, compatible with IgG aggregated binding to the Fc receptor; however, the MCHA values in the second incubation with horse ATG were similar to the nonaggregated IgG result.

To determine the specificity of horse ATG binding, sodium dodecyl sulfate (SDS) gel electrophoresis of platelet preparations and immunoblots with the normal horse serum and horse ATG were performed. These results are shown in figure 2. Normal horse serum showed no reactivity with any specific platelet glycoprotein. In contrast, two bands are noted with the horse ATG preparation. The major band is with a platelet glycoprotein of approximately 83,000 kd and the lighter staining band is with a glycoprotein of approximately 125–130 kd. The 83,000 kd band is the same molecular weight as glycoprotein \( V \); the second band has a molecular weight similar to glycoprotein \( \text{IIa} \).

<table>
<thead>
<tr>
<th>Platelet Source</th>
<th>MCHA†</th>
<th>MCHA Ratio</th>
</tr>
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<tbody>
<tr>
<td>HLA-typed pool</td>
<td>13.1</td>
<td>31.1</td>
</tr>
<tr>
<td>Donor 1 (A2,3; B60,39)</td>
<td>12.0</td>
<td>38.5</td>
</tr>
<tr>
<td>Donor 2 (A2,30; B35,7)</td>
<td>12.4</td>
<td>37.3</td>
</tr>
<tr>
<td>Donor 3 (A11, 24; B60,62)</td>
<td>11.3</td>
<td>35.0</td>
</tr>
</tbody>
</table>

*Human leukocyte-associated antigens.
†Mean value of region A.
‡Normal horse serum.
§Anti-thymocyte globulin.
PLATELET SPECIFIC ANTIBODIES

TABLE II
Effect of Platelet Pre-incubation
With Human Immunoglobulin Sources
on Anti-thymocyte Globulin

<table>
<thead>
<tr>
<th>Platelet Source</th>
<th>MCHA*</th>
<th>MCHA Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-ipay pool</td>
<td>7.1</td>
<td>30.1</td>
</tr>
<tr>
<td>HLA-ipay pool</td>
<td>6.9</td>
<td>31.6</td>
</tr>
<tr>
<td>HLA-ipay pool</td>
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</tr>
<tr>
<td>HLA-ipay pool</td>
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<td>28.3</td>
</tr>
<tr>
<td>HLA-ipay pool</td>
<td>12.9</td>
<td>28.4</td>
</tr>
</tbody>
</table>

*Mean value of region A.
†Normal horse serum.
§Anti-thymocyte globulin.
¶Human leukocyte-associated antigens.

Discussion

Anti-thymocyte globulin has a number of adverse effects associated with its use including thrombocytopenia. That this side effect may be on an immune basis was first suggested by Rosenberg and co-workers who proposed several possible mechanisms to account for the thrombocytopenia including platelet activation as a result of immune complex formation between the anti-thymocyte globulin and lymphocytes. They noted when horse ATG was added to platelet rich plasma in vitro, significant platelet aggregation and release took place. They suggested that immune complex formation was with a platelet membrane component rather than with another cell type. Subsequently Greco et al in a study of horse ATG interaction with lymphocytes, granulocytes, platelets, and erythroid bone marrow were able to demonstrate that horse ATG was equally reactive with distinct antigens on each type of cell but that the reaction with platelets was only of low intensity, i.e., mean intensity of fluorescence was minimal. This was true for six different lots of horse ATG tested. Wechter et al performed a variety of immune assays on 33 different lots of horse ATG including a complement fixation assay for anti-platelet antibodies. All of these lots showed anti-platelet reactivity.

Our studies confirm and extend these reports. Several features of this immune reaction are noteworthy: (1) previous sensitization is not required for the binding of horse ATG to platelet surface proteins since this occurred in the presence or absence of the donor’s plasma; (2) the interaction is dose-dependent and can be saturated implying there are specific receptor proteins on the platelet surface with which the immunoglobulin is binding; (3) platelet glycoproteins V and IIa appear to be the reactive antigens, at least with the lot of horse ATG employed in our studies. No hemostatic function is known for glycoprotein IIa. Glycoprotein V, however, may be the thrombin receptor on the surface of intact platelets. Other studies question the biological significance of this proposal since chymotrypsin digestion of glycoprotein

![Figure 2](image-url)

**Figure 2.** Transblot analysis following sodium dodecyl sulfate polyacrylamide gel electrophoresis of HLA-typed pooled platelet preparation. The electrophoretically separated platelet proteins were reacted with: (A) monoclonal mouse anti-human glycoprotein IIIa, (B) ATGAM, (C) normal horse serum.
V or blocking of this protein by specific antibody does not impair thrombin induced platelet aggregation; (4) the reaction is not inhibited by platelet antibody to other glycoproteins, i.e., IIIa nor by blocking platelet Fc receptors with aggregated human IgG.

These studies clearly demonstrate the presence of antiplatelet specific antibody in horse ATG preparation as was shown by the results with flow cytometric analysis. This assay, however, does not distinguish between reactivity with HLA antigens on the platelet surface versus reactivity with platelet specific antigens. The immunoblot studies document the latter but do not exclude the additional presence of anti-HLA reactivity against the platelet since HLA antigens can not be detected on this type of immunoblot analysis.

References