Studies on the Pathophysiology of Posttransfusion Purpura

By Thomas S. Kickler, Paul M. Ness, Jay H. Herman, and William R. Bell

Posttransfusion purpura typically occurs in PL\textsuperscript{A} negative blood recipients who have been previously immunized to the PL\textsuperscript{A} antigen. Following transfusion, severe thrombocytopenia develops with the formation of anti-PL\textsuperscript{A}. Since the patients' platelets lack the PL\textsuperscript{A} antigen, one would not expect this antibody to destroy autologous platelets. In this study we show that PL\textsuperscript{A} antigen exists in stored blood and can absorb to PL\textsuperscript{-} negative platelets making them PL\textsuperscript{A} reactive. Incubating PL\textsuperscript{A} (-) platelets with ultracentrifuged plasma from PL\textsuperscript{A} (+) blood donors allowed anti-PL\textsuperscript{A} to bind to PL\textsuperscript{A} (-) platelets. Control plasma from PL\textsuperscript{A} (-) blood donors did not lead to anti-PL\textsuperscript{A} binding. Using an inhibition assay, we showed that stored blood contains PL\textsuperscript{A} material that was not removed by ultracentrifugation.

The syndrome of posttransfusion purpura (PTP) was initially described by Shulman and coworkers.\textsuperscript{1} This disorder typically occurs in patients whose platelets lack the platelet-specific antigen PL\textsuperscript{A}, although other antigens have also been incriminated. Van Loghem and coworkers had originally characterized this platelet antigen system and named it Zw.\textsuperscript{2} Thrombocytopenia occurs after transfusion with PL\textsuperscript{A} positive blood in individuals previously sensitized by pregnancy or transfusion. With the development of thrombocytopenia, anti-PL\textsuperscript{A} is found in the patient's blood. The perplexing question is why the patient's own PLA\textsuperscript{A} (-) platelets are destroyed. One would not expect the platelet alloantibody to destroy autologous platelets since they lack the PL\textsuperscript{A} antigen.

Various mechanisms have been proposed to explain the induction of PTP. These include the production of autoantibodies\textsuperscript{3} or immune complex-mediated platelet destruction as occurs in drug-induced immune thrombocytopenia.\textsuperscript{4} A third explanation is that transfused antigenic material adheres to the PL\textsuperscript{A} (-) platelets permitting the binding of antibody with resultant platelet destruction. This possibility is suggested by the reported ability of platelets to absorb blood group antigens and HLA antigens.\textsuperscript{5,4}

This report presents experimental results supporting the hypothesis that PL\textsuperscript{A} (-) platelets can become PL\textsuperscript{A} reactive by absorbing transfused PL\textsuperscript{A} reactive material.

**MATERIALS AND METHODS**

**Antisera.** Anti-PL\textsuperscript{A} was obtained from a PL\textsuperscript{A} (-) woman 2 months following recovery from PTP. Another example of anti-PL\textsuperscript{A} was obtained from a mother of a child with alloimmune thrombocytopenia. These anti-PL\textsuperscript{A} sera reacted at a titer of 1:256 with PL\textsuperscript{A} (+) platelets.

**Platelets.** Group 0, PL\textsuperscript{A} (-) platelets were prepared from EDTA anticoagulated blood.\textsuperscript{8} The platelets were then washed four times with Tyrode's-EDTA, 3% BSA buffer. The platelet count was adjusted to 200,000/mm\textsuperscript{3}.

**Radiolabeled antiglobulin test to measure anti-PL\textsuperscript{A} binding.** Anti-PL\textsuperscript{A} binding to platelets was measured using a radiolabeled antiglobulin technique.\textsuperscript{9,10} The anti-PL\textsuperscript{A} binding is measured using affinity purified \textsuperscript{125I} goat antihuman IgG labeled by the chloramine T method.\textsuperscript{11}

**Transfer of PL\textsuperscript{A} reactivity to PL\textsuperscript{A} (-) platelets.** To determine if PL\textsuperscript{A} (-) platelets can absorb PL\textsuperscript{A} antigenic material from stored blood, the following were done. Plasma was obtained from units of CPD-A\textsubscript{1} anticoagulated PL\textsuperscript{A} (+) whole blood that had been stored 5 to 7 days. The plasma were centrifuged at 100,000 g for 30 minutes to remove any particulate material. Two mL of the plasma were incubated with 4 x 10\textsuperscript{5} platelets from PL\textsuperscript{A} (-) individuals for 12 hours at 22 °C. Following the incubation, platelets were washed using Tyrodes-BSA buffer.

These platelets were then assayed for their ability to bind anti-PL\textsuperscript{A} using the indirect radiolabeled antiglobulin test. For negative controls, PL\textsuperscript{A} (-) platelets were incubated with plasma obtained from stored whole blood from a PL\textsuperscript{A} (-) donor, or with buffer. For an additional control, red cells were substituted for platelets to exclude the possibility that observed anti-PL\textsuperscript{A} binding was related to trapping of platelet membrane debris or microparticles.

**Inhibition assay to measure PL\textsuperscript{A} antigenic material in stored blood.** To determine the frequency with which stored blood contains a plasma form of PL\textsuperscript{A} antigenic material, an inhibition assay to measure PL\textsuperscript{A} antigen was devised. A total of 25 stored units were tested.

The test plasma was centrifuged at 100,000 g for 30 minutes. One mL of this plasma was incubated for 30 minutes at 37 °C with 1 mL of anti-PL\textsuperscript{A} that had been diluted to a titer of 1:128. The mixture was then added to 2 x 10\textsuperscript{5} platelets to determine the amount of free anti-PL\textsuperscript{A} remaining that could bind to the platelets as determined by the radiolabeled antiglobulin test. The inhibition of the anti-PL\textsuperscript{A} binding after incubation with test plasma from stored PL\textsuperscript{A} (+) positive blood was compared to the inhibition when plasma from a PL\textsuperscript{A} (-) donor was used. The source of this plasma was from an untransfused PL\textsuperscript{A} (-) donor. The lack of inhibitory activity of the PL\textsuperscript{A} (-) plasma was also confirmed by comparison to the inhibition caused by buffer.

**Western blotting.** To characterize the material binding to PL\textsuperscript{A} (-) platelets, we analyzed the converted platelets using Western blotting. After transferring PL\textsuperscript{A} positivity to negative platelets, 2 x 10\textsuperscript{5} platelets were solubilized in 3% SDS preparation for SDS-polyacrylamide gel electrophoresis according to the method of McMillan.\textsuperscript{12}

SDS-Polyacrylamide gel electrophoresis. The solubilized platelets were electrophoresed using the method of Laemmli.\textsuperscript{13} Slab gels

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of 1.5 mm thickness and 6% acrylamide were used with a 3% stacking gel. Molecular weight standards (Sigma, St. Louis, Mo) were run with each experiment.

Protein transfer to nitrocellulose paper. Proteins were electrophoretically transferred from gels to nitrocellulose paper using the method of Towbin. Complementarity of transfer was documented by fast green staining of the nitrocellulose.

Incubation of radiolabeled IgG with nitrocellulose immobilized proteins. Prior to incubation with anti-PLAI serum, the immobilized proteins on the nitrocellulose strips were immersed for three hours in 3% BSA and 10 mmol/L Tris, 15 mol/L NaCl, 1% Tween 20. The anti-PLAI serum was incubated one hour at 25 °C with the nitrocellulose paper. After washing the strips, with 3% BSA and 1% Tween 20 in mmol/L Tris, they were incubated with 125I anti-IgG for one hour followed by additional washing to remove unbound radioactivity.

RESULTS

Conversion experiments. The ability of PLAI (−) platelets to acquire the capacity to bind anti-PLAI is shown in Table 1. When PLAI (−) platelets were incubated with plasma from four different units of stored PLAI (+) blood, anti-PLAI binding was detected in all cases. The plasmas used as a source of PLAI antigen were from 1-, 2-, 7-, and 10-day-old stored blood. Anti-PLAI binding was 1.8 to 2.7 times greater than the negative controls. However, the amount of measured anti-PLAI binding to the PLAI (−) platelets after incubation did not equal the amount of anti-PLAI binding to PLAI (−) platelets. Since the anti-PLAI did not react with PLAI (−) platelets until incubated with plasma from PLAI (+) plasma, PLAI (−) platelets must acquire the ability to bind anti-PLAI from some factor present in the plasma. Since all experiments used ABO compatible platelets and plasma and since PLAI (−) plasma did not promote anti-PLAI binding, the results show that PLAI (−) platelets can absorb PLAI antigenic material.

When red cells are substituted for platelets, no binding of anti-PLAI to the red cells is observed. This suggests that the anti-PLAI binding was unrelated to trapping of platelet membrane debris or microparticles. When anti-PLAI from a patient with neonatal alloimmune thrombocytopenia was used instead of anti-PLAI from a patient who recovered from posttransfusion purpura, similar results were obtained.

Inhibition assay results. To determine if most units of stored blood from PLAI (+) donors contain PLAI antigenic material in the plasma, we studied 25 randomly selected units of stored blood and analyzed ultracentrifuged plasma using an inhibition assay. The results of this analysis are shown in Table 2. Negative controls consisted of plasma from a PLA1 (−) donor who had never been immunized, and freshly collected plasma from PLAI (+) donors. In Table 3, the results of a representative inhibition assay are shown.

All test plasmas obtained from stored units of blood promoted greater than 54% inhibition in anti-PLAI binding to PLAI (+) platelets. The amount of inhibition was similar for short-term stored units (one to three days) and units stored for longer time (seven to ten days). The specificity of the inhibition assay was shown by the lack of inhibition when plasma from a PLAI (−) donor was used. These results show that all stored units contain material that can bind to PLAI (−) platelets as described above.

Western blotting results. Converted platelets were also studied using Western blotting to identify PLAI antigenic material. Plasma capable of promoting anti-PLAI binding, as measured by the radiolabeled antiglobulin test, was incubated with PLAI (−) platelets. A control consisted of using plasma from a PLAI (−) donor. After incubating these plasmas with PLAI (−) platelets, the platelets were solubilized and run in parallel with PLAI (+) and PLAI (−) platelets. The resultant autoradiograph is shown in Fig 1. One can see that when plasma from a PLAI (+) donor is used, a band at 95,000 daltons is observed which is not detected when the PLAI (−) platelets are incubated with plasma from a PLAI (−) donor. This band corresponds to the reported molecular weight of PLAI. The nonspecific reactivity at 210,000, present in all lanes, is enhanced by incubation with plasma (lanes A and B), probably the result of absorbed plasma IgG. When the Western blot was developed using normal AB plasma in place of anti-PLAI plasma, no bands in the region corresponding to the 95,000 Dalton band were observed. These results provide further support that PLAI antigenic material is present in plasma.

DISCUSSION

Central to the mystery of posttransfusion purpura is why patients destroy their own platelets that lack the antigen to which they have been sensitized. Shulman et al have pointed out the similarity to drug-induced purpura, where there is an
Table 3. Results of a Representative Inhibition Assay

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<th>Counts Per Minute of Measured Anti-PLA\textsuperscript{i} Binding (experiment performed in triplicate)</th>
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<tbody>
<tr>
<td>Plasma from PL\textsuperscript{A1}(+) Donor</td>
<td>6,045 (62%)</td>
</tr>
<tr>
<td>Plasma from PL\textsuperscript{A1}(−) Donor</td>
<td>4,581 (62%)</td>
</tr>
<tr>
<td>Buffer</td>
<td>15,615</td>
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The inhibition assay used plasma centrifuged at 100,000 g for 30 minutes; this was incubated with anti-PLA\textsuperscript{i}, diluted 1:128, for 30 minutes. PL\textsuperscript{A1}(+) platelets were then added and the amount of anti-PLA\textsuperscript{i} binding was determined using the radiolabeled antiglobulin test. The percent inhibition is given in parenthesis.

abrupt onset of thrombocytopenia, followed by recovery despite persistence of the inciting antibody. These investigators proposed that PLA\textsuperscript{i} antigen-antibody complexes bind to platelets in a similar fashion to drug purpura as one possible mechanism for PTP.

Serologic studies during the acute phase of PTP have demonstrated the presence of increased levels of IgG on the surface of the patient’s platelets.\textsuperscript{10,15} Following elution of this IgG activity, Pegels showed anti-PLA\textsuperscript{i} specificity with strong reactivity against known PL\textsuperscript{A1}(+) platelets. They also observed weak reactivity with PL\textsuperscript{A1}(−) autologous platelets from patients in remission. They interpreted these results to indicate the presence of PL\textsuperscript{A1}-anti-PLA\textsuperscript{i} complexes binding to platelets. These results, however, are in conflict with the general concept that eluates of cell-associated immune complexes are nonreactive whereas cell-associated antibodies will demonstrate reactivity.\textsuperscript{16}

Morrison and Mollison have proposed that a second antibody with autoantibody reactivity is also produced in PTP which reacts with PLA\textsuperscript{i} negative platelets.\textsuperscript{5} In 3 patients we studied, autoantibody was not found using acute plasma testing against autologous platelets following recovery.\textsuperscript{17} Slichter also reported one patient in whom autoantibodies were not found.\textsuperscript{18}

The third proposed mechanism of PLA\textsuperscript{i} antigenic material binding to PLA\textsuperscript{i}(−) platelets with subsequent destruction by the alloantibody is consistent with reported properties of platelets. Platelets may absorb many substances including proteins, blood group antigens, and HL antibodies.\textsuperscript{5,7} Therefore, it is possible that PLA\textsuperscript{i} antigen may absorb to PLA\textsuperscript{i}(−) platelets if the antigen is transfused to a patient. To support this hypothesis, PLA\textsuperscript{i} antigen must be present in soluble form in stored blood.

It is known that few intact platelets remain after 24 to 48 hours of storage at 4 °C. Therefore, antigenic material may exist as particulate material and possibly soluble forms. Despite meticulous blood collection, platelet deterioration occurs with storage. Ultrastructural alterations are consistent with loss of surface membrane integrity.\textsuperscript{19} Various investigators have also described loss of platelet glycoproteins from the surface of stored platelets resulting from hydrolysis or protease activity.\textsuperscript{20} These studies support the possibility that antigenic material may exist in stored plasma.

Previous attempts by Shulman and coworkers to detect a soluble form of PLA\textsuperscript{i} using a complement fixation technique were unsuccessful.\textsuperscript{17} These investigators could detect large amounts of particulate PLA\textsuperscript{i} antigen in plasma but this material was removed with filtration. Similar experiments by Aster using a \textsuperscript{51}Cr release assay also demonstrated PLA\textsuperscript{i} reactive material in serum which was removed by centrifugation.\textsuperscript{21} More recently, Warejeka and coworkers have demonstrated PLA\textsuperscript{i} reactivity that did not sediment at 100,000 x g for 60 minutes.\textsuperscript{22}

Our results show that following ultracentrifugation, PLA\textsuperscript{i} antigenic material is present in a form that can convert PLA\textsuperscript{i} negative platelets to a PLA\textsuperscript{i} positivity. In addition, using an inhibition assay, PLA\textsuperscript{i} antigen is present in ultracentrifuged plasma from all units of stored blood that were examined. The Western blotting experiments provide further verification that PLA\textsuperscript{i} antigenic material can be found in plasma from a PLA\textsuperscript{i} positive donor.

PLA\textsuperscript{i} antigen has been shown to be present on glycoprotein IIIa.\textsuperscript{22} The molecular weight has been estimated to be 95,000 ± 10,000 daltons.\textsuperscript{23} Although the converted platelets appear to have weaker reactivity than the PL\textsuperscript{A1}(+) control, they clearly acquired antigenic material of this molecular weight from the PL\textsuperscript{A1}(+) plasma.

Our studies suggest the following possible mechanism for posttransfusion purpura. PLA\textsuperscript{i} antigenic material from stored blood can absorb to PLA\textsuperscript{i} negative platelets. Upon transfusion, this material may bind to the patient’s PLA\textsuperscript{i} negative platelets. With the formation of anti-PLA\textsuperscript{i} following an anamnestic immune response, the patient’s platelets are destroyed leading to thrombocytopenia.

This explanation based on our in vitro experiments must also be evaluated in terms of clinical observations. Some patients with PTP may have thrombocytopenia for 4 weeks.
This would imply that sufficient antigen must be present for the duration of the thrombocytopenia. Intuitively, one would not expect prolonged survival of a transfused antigen unless there is a recycling process. Alternatively it is conceivable that, following the development of thrombocytopenia, the relative amount of antigen to the smaller number of platelets being produced is sufficient to maintain the thrombocytopenia. Shulman first proposed this theory to explain the prolonged course of thrombocytopenia. To verify if this is the situation, further studies are needed to determine if PL$^A$ antigenic material is detectable in the patient’s serum. Successful attempts at measuring circulating antigen have not been reported in patients with PTP.

Also, careful clinical observations are needed to determine if, in fact, the prolonged thrombocytopenia seen in some patients is related to PTP etiologic factors versus complicating clinical problems that impair thrombopoiesis.

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