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

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

Various mechanisms have been proposed to explain the induction of PTP. These include the production of autoantibodies or immune complex-mediated platelet destruction as occurs in drug-induced immune thrombocytopenia.\(^{4}\) A third explanation is that transfused antigenic material adheres to the PL\(^{+}\) (-) 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.\(^{5}\)\(^{4}\)

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

MATERIALS AND METHODS

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

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

**Radiolabeled antiglobulin test to measure anti-PLAI binding.** Anti-PL\(^{+}\) binding to platelets was measured using a radiolabeled antiglobulin technique.\(^{8}\) The anti-PL\(^{+}\) binding is measured using affinity purified \(1\) goat antihuman IgG labeled by the chloramine T method.\(^{11}\)

**Transfer of PLAI reactivity to PLAI(-) platelets.** To determine if PL\(^{+}\) (-) platelets can absorb PL\(^{+}\) antigenic material from stored blood, the following were done. Plasma was obtained from units of CPD-A1 anticoagulated PL\(^{+}\) (+) 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\(^{9}\) platelets from PL\(^{+}\) (-) individuals for 12 hours at 22 °C. Following the incubation, platelets were washed using Tyrode's-BSA buffer.

These platelets were then assayed for their ability to bind anti-PL\(^{+}\) using the indirect radiolabeled antiglobulin test. For negative controls, PL\(^{+}\) (-) platelets were incubated with plasma obtained from stored whole blood from a PL\(^{+}\) (-) donor, or with buffer.

For an additional control, red cells were substituted for platelets to exclude the possibility that observed anti-PL\(^{+}\) binding was related to trapping of platelet membrane debris or microparticles. An inhibition assay to measure PLAI antigenic material in stored blood. To determine the frequency with which stored blood contains a plasma form of PL\(^{+}\) antigenic material, an inhibition assay to measure PL\(^{+}\) 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\(^{+}\) that had been diluted to a titer of 1:128. The mixture was then added to 2 x 10\(^{9}\) platelets to determine the amount of free anti-PL\(^{+}\) remaining that could bind to the platelets as determined by the radiolabeled antiglobulin test. The inhibition of the anti-PL\(^{+}\) binding after incubation with test plasma from stored PL\(^{+}\) (+) positive blood was compared to the inhibition when plasma from a PL\(^{+}\) (-) donor was used. The source of this plasma was from an untransfused PL\(^{+}\) (-) donor. The lack of inhibitory activity of the PL\(^{+}\) (-) plasma was also confirmed by comparison to the inhibition caused by buffer.

**Western blotting.** To characterize the material binding to PL\(^{+}\) (-) platelets, we analyzed the converted platelets using Western blotting. After transferring PL\(^{+}\) (+) positive platelets, 2 x 10\(^{9}\) platelets were solubilized in 3% SDS preparation for SDS-polyacrylamide gel electrophoresis according to the method of McMillan.\(^{12}\) SDS-Polyacrylamide gel electrophoresis. The solubilized platelets were electrophoresed using the method of Laemelli.\(^{13}\) Slab gels were stained with Coomassie blue.

From The Johns Hopkins University School of Medicine, Departments of Laboratory Medicine and Medicine, Baltimore. Submitted Oct 14, 1985; accepted March 12, 1986.

Address reprint requests to Thomas S. Kickler, MD, Associate Director, Blood Bank, The Johns Hopkins Hospital, 600 N. Wolfe St., Baltimore, MD 21205.

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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 electro-phoretically transferred from gels to nitrocellulose paper using the method of Towbin. Completeness of transfer was documented by fast green staining of the nitrocellulose.

Incubation of radiolabeled IgG with nitrocellulose immobilized proteins. Prior to incubation with anti-PLA serum, the immobilized proteins on the nitrocellulose strips were immersed for three hours in 3% BSA and 10 mmol/L Tris, pH 7.2. The anti-PLA 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 PLAN(-) platelets to acquire the capacity to bind anti-PLA is shown in Table 1. When PLAN(-) platelets were incubated with plasma from four different units of stored PLAIN(+) blood, anti-PLA binding was detected in all cases. The plasmas used as a source of PLAIN antigen were from 1-, 2-, 7-, and 10-day-old stored blood. Anti-PLA binding was 1.8 to 2.7 times greater than the negative controls. However, the amount of measured anti-PLA binding to the PLAIN(-) platelets after incubation did not equal the amount of anti-PLA binding to PLAIN(+) platelets. Since the anti-PLA did not react with PLAIN(-) platelets until incubated with plasma from PLAIN(+) plasma, PLAIN(-) platelets must acquire the ability to bind anti-PLA from some factor present in the plasma. Since all experiments used ABO compatible platelets and plasma and since PLAIN(-) plasma did not promote anti-PLA binding, the results show that PLAIN(-) platelets can absorb PLAIN antigenic material.

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

Inhibition assay results. To determine if most units of stored blood from PLAIN(+) donors contain PLAIN 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 PLAIN(-) donor who had never been immunized, and freshly collected plasma from PLAIN(+) 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-PLA binding to PLAIN(+) 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 PLAIN(-) donor was used. These results show that all stored units contain material that can bind to PLAIN(-) platelets as described above.

Western blotting results. Converted platelets were also studied using Western blotting to identify PLAIN antigenic material. Plasma capable of promoting anti-PLA binding, as measured by the radiolabeled antiglobulin test, was incubated with PLAIN(-) platelets. A control consisted of using plasma from a PLAIN(-) donor. After incubating these plasmas with PLAIN(-) platelets, the platelets were solubilized and run in parallel with PLAIN(+) and PLAIN(-) platelets. The resultant autoradiograph is shown in Fig 1. One can see that when plasma from a PLAIN(+) donor is used, a band at 95,000 daltons is observed which is not detected when the PLAIN(-) platelets are incubated with plasma from a PLAIN(-) donor. This band corresponds to that observed with native PLAIN(+) platelets and is the reported molecular weight of PLAIN. The nonspecific activity 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-PLA plasma, no bands in the region corresponding to the 95,000 Dalton band were observed. These results provide further support that PLAIN 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
abrupt onset of thrombocytopenia, followed by recovery despite persistence of the inciting antibody. These investigators proposed that PLAl 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. Following elution of this IgG activity, Pegels showed anti-PLAl specificity with strong reactivity against known PLAl (+) platelets. They also observed weak reactivity with PLAl (-) autologous platelets from patients in remission. They interpreted these results to indicate the presence of PLAl-anti-PLAl 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.

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

The third proposed mechanism of PLAl antigenic material binding to PLAl (-) platelets with subsequent destruction by the alloantibody is consistent with reported properties of PLAl. Platelets may absorb many substances including proteins, blood group antigens, and HLA antigens. Therefore, it is possible that PLAl antigen may absorb to PLAl (-) platelets if the antigen is transfused to a patient. To support this hypothesis, PLAl 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 exists as particulate material and possibly soluble forms.

Table 3. Results of a Representative Inhibition Assay

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<thead>
<tr>
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<th>Counts Per Minute of Measured Anti-PLAl Binding (experiment performed in triplicate)</th>
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<tbody>
<tr>
<td>Plasma from PLAl(+) Donor</td>
<td>6,045 (62%)</td>
</tr>
<tr>
<td>Plasma from PLAl(-) Donor</td>
<td>15,885</td>
</tr>
<tr>
<td>Buffer</td>
<td>15,053</td>
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The inhibition assay used plasma centrifuged at 100,000 g for 30 minutes; this was incubated with anti-PLAl, diluted 1:128, for 30 minutes. PLAl(+) platelets were then added and the amount of anti-PLAl binding was determined using the radiolabeled antiglobulin test. The percent inhibition is given in parenthesis.

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

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

PLAl antigen has been shown to be present on glycoprotein IIIa. The molecular weight has been estimated to be 95,000 ± 10,000 daltons. Although the converted platelets appear to have weaker reactivity than the PLAl(+) control, they clearly acquired antigenic material of this molecular weight from the PLAl(+) plasma.

Our studies suggest the following possible mechanism for posttransfusion purpura. PLAl antigenic material from stored blood can absorb to PLAl negative platelets. Upon transfusion, this material may bind to the patient’s PLAl negative platelets. With the formation of anti-PLAl 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-A1 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.

REFERENCES

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