Posttransfusion Purpura: Conversion of PL^{A1}-Negative Platelets to the PL^{A1}-Positive Phenotype by Stored Plasma is not due to the Presence of Soluble PL^{A1} Antigen (43133)

W. C. EHMANN, A. DANCIS, R. FERZIGER, AND S. KARPATKIN
Department of Medicine, New York University Medical School, New York, New York 10016

Abstract. **Three previous articles have reported a PL^{A1} antigen in the plasma of stored blood which is capable of binding to PL^{A1}-negative platelets in the presence of divalent cations, rendering them PL^{A1} positive. Such a mechanism could explain the enigma of posttransfusion purpura (PTP), i.e., severe thrombocytopenia in a healthy subject with PL^{A1}-negative platelets secondary to the infusion of blood containing PL^{A1}-positive platelets. We find that the PL^{A1} antigen of stored blood is due to the presence of platelet fragments which can be removed by centrifugation and that divalent cation-chelating agents play no role in the apparent binding of these fragments to platelets. The apparent conversion of PL^{A1}-negative platelets to the PL^{A1}-positive phenotype by incubation with stored plasma from a PL^{A1}-positive subject is due to the cosedimentation of platelet fragments with the platelets. No soluble PL^{A1} antigen was found in the plasma of five patients with acute posttransfusion purpura.** [P.S.E.B.M. 1990, Vol 195]

Posttransfusion purpura (PTP) is a well-described disorder in which patients (usually female) become profoundly thrombocytopenic 5–7 days after a blood transfusion. The transfused blood contains a platelet antigen which the patient lacks, usually PL^{A1} (1–3). Introduction of the antigen produces an anamnestic response. There is often a history of prior exposure via pregnancy or blood transfusion. Antibody-mediated destruction of transfused platelets with the antigen as well as autologous platelets lacking the antigen produces severe thrombocytopenia. The explanation for this phenomenon has remained elusive.

Three publications (4–6) have reported or implied that the PL^{A1} epitope (which resides on platelet glycoprotein GPIIIa (7)) may exist in soluble form in transfused blood. It has been suggested that the soluble PL^{A1} epitope in transfused blood binds to PL^{A1}-negative platelets (also designated PL^{A2}), thus converting them to a PL^{A1}-positive phenotype (4–6). These platelets could then be recognized by the anti-PL^{A1} antibodies, and rapidly cleared.

Received March 16, 1990. [P.S.E.B.M. 1990, Vol 195]
Accepted May 22, 1990.

0037-9727/90/1952-0192$2.00/0

We studied this model using (i) a high titer anti-PL^{A1} antibody from a patient with PTP (8), (ii) a monoclonal antibody with stronger reactivity for PL^{A1}-positive platelets than negative platelets, and (iii) a monoclonal antibody against platelet membrane GPIIIa which reacts equally with PL^{A1}-positive and negative platelets, which we have produced (8). We found that neither the PL^{A1} epitope nor GPIIIa is present in soluble form in the plasma or platelet preparations of PL^{A1}-positive subjects. We also determined that the apparent *in vitro* conversion of PL^{A1}-negative to PL^{A1}-positive platelets after addition of stored plasma is due to the cosedimentation of PL^{A1}-positive platelet fragments with PL^{A1}-negative platelets.

Materials and Methods

Materials. Chemicals, reagents, staphylococcal protein A sepharose beads, Ponceau red stain, goat anti-mouse Ig, and goat anti-human IgG (γ chain specific) were obtained from Sigma Chemical, St. Louis, MO, unless otherwise noted. CPDA-1 anticoagulant (citrate, phosphate, dextrose pH 5.7) was obtained from Fenwal Laboratories, Division of Travenol Laboratories, Inc., Deerfield, IL.

Platelet Preparations. Blood was collected into a 0.8% citric acid, 2.2% trisodium citrate dihydrate, and 2.45% dextrose (ACD) solution (Travenol) at an ACD

to blood ratio of 1:6. Platelet-rich plasma (PRP) was prepared by centrifugation at 100*g* for 7 min. PRP from this blood was spun at 2500*g* for 30 min and the platelet pellet was suspended in 10 times its volume of 1% ammonium oxalate. After 10 min the platelets were spun at 2500*g* for 10 min and washed twice with large volumes of a modified human Ringer's solution containing 2 m*M* EDTA (9).

For experiments in which we sought "soluble PL^{A1}" antigen, washed platelets were resuspended in their original plasma volume of a modified human Ringer's solution containing 2 m*M* EDTA and stored at 4°C for 5 days. These preparations were then centrifuged for 15 min at 2500*g*, platelets were removed, and the supernatant was centrifuged at 100,000*g* for 1 or 2 hr to remove particulate matter. The respective supernatants were run on 10% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) for immunoblotting.

For experiments in which EDTA was not used, platelets were collected into ACD and treated with aspirin to a final concentration of 0.1 m*M* acetylsalicylic acid. The PRP was then spun as described above and resuspended in 1% ammonium oxalate. The subsequent washings were performed by using a washing fluid (10) to which 2 μM PGE_1 (Sigma Chemical) and 100 μg/ml of apyrase (Sigma) were added. After incubation these platelets were then centrifuged at the designated gravity for 45 min, and equal volumes of each of the supernatants were run on SDS-PAGE.

For the experiments in which platelets were incubated with plasma or serum, the washed platelet suspension was adjusted to 200,000/μl and divided into aliquots. The aliquots were then spun at 2500*g* for 30 min, the supernatant was removed, and the platelet button was resuspended in 2 ml of one of the various plasma or serum samples at their original washed platelet concentration. Each aliquot was then agitated gently overnight at 22°C. The platelets were then spun and washed three times as described above and lysed with 200 μl of 10% SDS in 60 m*M* Tris, pH 6.8 (11) (lysing buffer). After vortexing, an equal volume of sample buffer was added, and 100 μl of this solution were placed in each lane of a 10% SDS-PAGE. Known PL^{A1}-positive and negative platelets were similarly prepared and run on the same gel.

Some washed platelet preparations were stored frozen after addition of dimethyl sulfoxide, 5% final concentration. The platelets were aliquoted and left at 22°C for 1 hr and then stored at −70°C until needed. For use, the platelets were thawed at room temperature and washed twice with the washing solution. PL^{A1}-positive platelets, stored under these conditions, were shown to retain PL^{A1} reactivity with the various antibodies.

Antibodies. Monoclonal antibodies to platelet surface glycoprotein IIIa were prepared as described previously (8). DEK-16 is an IgG1-κ murine monoclonal antibody to GPIIIa; it reacts equally with PL^{A1}-positive and PL^{A1}-negative platelets. DEK-1 is an IgM-κ murine monoclonal antibody that reacts strongly with PL^{A1}-positive platelets and weakly with PL^{A1}-negative platelets on immunoblot at a 100-kDa band. The polyclonal antibody, BE, was prepared from a patient with posttransfusion purpura.

This patient was a 69-year-old female (with previous pregnancy) who, during a coronary artery bypass-grafting procedure, received 9 units of packed red blood cells. Five days postoperatively, her platelet count was normal. Three days later she had petechiae, gingival bleeding, and a platelet count of 18,000/μl. A bone marrow aspirate revealed increased megakaryocytes. The patient was treated initially with steroids and intravenous γ-globulin, to which she did not respond. She was then plasmapheresed and had an immediate and sustained response. Her platelets obtained during remission were subsequently found to be PL^{A1} negative. Plasma from her first plasmapheresis was frozen in aliquots. For use in immunoblotting, the plasma was defibrinated by adding calcium and incubating at 37°C for 1 hr. After centrifugation, the serum was agitated overnight with an equal volume of saturated ammonium sulfate. The precipitate was washed twice with a 50% solution of ammonium sulfate dissolved in 0.1 *M* phosphate buffer-0.154 *M* NaCl, pH 7.4 (phosphate-buffered saline), and the residual precipitate-salt mixture dissolved in a small quantity of distilled water. This solution was then dialyzed against water for 24 hr, followed by phosphate-buffered saline-0.025% sodium azide for 24 hr. For immunoblotting, this antibody was used at a dilution of 1/5000 in BLOTTO (see below) (12).

DEK-16 antibody was iodinated using IODO-GEN (Pierce, Rockford IL).

Plasma. Plasma was obtained from blood of PL^{A1}-positive and negative subjects by collecting into ACD at an anticoagulant to blood ratio of 1:6. After centrifugations at 280*g*, then 2500*g*, the platelet-poor plasma was either used directly or stored at −20°C until thawed at 37°C prior to use. Serum was obtained from a PL^{A1}-positive donor by collection of blood into a glass tube, incubation at 37°C for 1 hr, and centrifugation at 2500*g*. Stored blood was obtained from a PL^{A1}-positive donor by withdrawal into CPDA-1 (1:6), gentle mixing, and maintenance at 4°C for 7 days. Platelet-poor plasma was prepared from this blood by centrifugation as described above. One half of this platelet-poor plasma was then centrifuged at 100,000*g* for 1 or 2 hr, and the supernatant was collected.

Immunoblots. Whole platelet lysates were prepared by solubilization of washed platelets in 10% SDS in 60 m*M* Tris buffer (pH 6.8), or in 1% Triton X-100 in 0.15 *M* NaCl-0.01 *M* Tris buffer (pH 7.4). Platelet

membrane preparations or whole platelet lysates were electrophoresed on 10% SDS-PAGE gels and then transferred to nitrocellulose paper for immunoblotting as described previously (8), employing either polyclonal anti-PL^{A1} antibody at a dilution of 1/5,000 or monoclonal antibodies DEK-1 or DEK-16 at dilutions of 1/50 to 1/1,000 in BLOTTO for 2 hr on a shaker at 37°C.

Results

Figure 1 (Lanes A and B) demonstrates that there are equal amounts of the PL^{A1} epitope in the 2500*g* supernatant of washed platelets incubated for 1 week with or without EDTA. Lanes C and D show that centrifugation at 100,000*g* eliminated all of the detectable PL^{A1} antigenic activity. Similar results were seen with other experiments (data not shown).

Figure 2 reflects the results of incubating PL^{A1}-negative platelets with various plasma and serum samples. Panel A was developed using DEK-1, a monoclonal antibody that reacts preferentially with the PL^{A1} epitope and weakly with the PL^{A1}-negative epitope. Lane 1 shows the small amount of reactivity seen with DEK-1 on PL^{A1}-negative platelets and lane 6 the reactivity of PL^{A1}-positive platelets. The 100-kDa band is GPIIIa (8). The 120-kDa band has recently been described and thought to be either a posttranslational precursor of GPIIIa or a homologous glycoprotein (8). The band above the 120-kDa region is an artifact seen because of cross-reactivity of the rabbit anti-mouse IgG antibody with the platelet preparation, presumably to platelet IgG and/or Fc receptors (data not shown). Lane 2 shows no increase in PL^{A1} activity when PL^{A1}-negative platelets were incubated with plasma from a PL^{A1}-negative subject. Lane 3 demonstrates no increase in PL^{A1} activity when PL^{A1}-negative platelets were incubated with plasma from 7-day stored blood from a PL^{A1}-positive subject, if the plasma was spun at 100,000*g* for 2 hr. In most experiments, similar results were noted after 1 hr of centrifugation at 100,000*g*. In one experiment a faint band of PL^{A1} reactivity was present at 1 hr but was gone at 2 hr of centrifugation. In contrast, the PL^{A1}-negative platelets in Lane 4 were incubated with the same plasma centrifuged only at 2500*g* for 15 min. This clearly shows PL^{A1} reactivity almost as strong as that on PL^{A1}-positive platelets. Lane 5 demonstrates no effect of serum obtained from a PL^{A1}-positive subject, indicating absence of platelet fragments which were presumably trapped within the clot. These results were confirmed by developing an identical immunoblot with BE, a polyclonal anti-PL^{A1} antibody, shown in Panel B. The numerous bands above the 120-kDa region as well as the two faint bands above and below the 100-kDa region are artifacts seen because of cross-reactivity of the goat anti-human IgG antibody with the platelet preparation, presumably to platelet IgG and/or Fc receptors (data not shown). As in Panel A, the conversion of PL^{A1}-negative platelets to PL^{A1}-positive platelets only occurs if the plasma from the PL^{A1}-positive subject is spun at low speed for a

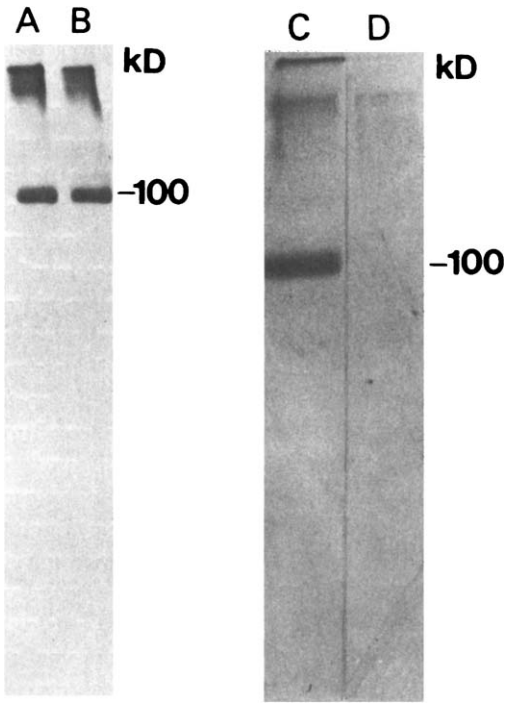

Figure 1. Immunoblot of supernatants of stored washed platelets. Platelets were washed and incubated in human Ringer's solution (3 × 10^8/ml) for 5 days with or without 2 m*M* EDTA, supernatants obtained by centrifuging at 2500*g* for 10 min, and 100 μl applied to SDS-PAGE (Lane A with EDTA, Lane B without EDTA). Lanes C and D show a similar preparation incubated with 2 m*M* EDTA, but after centrifugation at 2500*g* for 10 min (Lane C) and 100,000*g* for 1 hr (Lane D). All lanes were transferred to nitrocellulose and developed with BE, a polyclonal anti-PL^{A1} antibody.

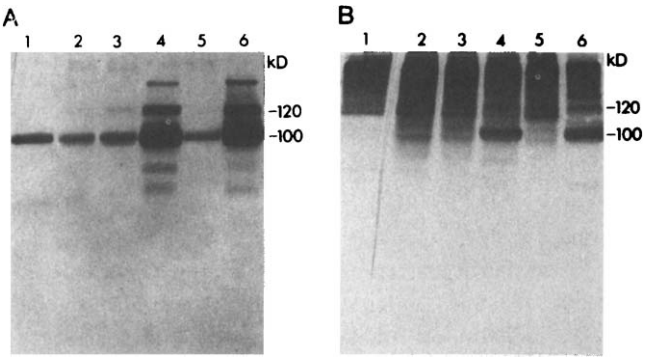

Figure 2. Immunoblots of PL^{A1}-negative platelet incubation experiments. Each lane contains 1 × 10^7 platelets applied in 100 μl. Panel A was developed with DEK-1, a monoclonal antibody to the PL^{A1} epitope. Panel B was developed with BE, a human polyclonal anti-PL^{A1} antibody from a patient with PTP. Lane 1 contains PL^{A1}-negative platelets, lane 6 PL^{A1}-positive platelets. Lanes 2 through 5 are PL^{A1}-negative platelets incubated with: plasma from a PL^{A1}-negative subject (Lane 2), plasma from blood stored for 1 week from a PL^{A1}-positive subject centrifuged at 100,000*g* for 2 hr (Lane 3), plasma from stored blood from a PL^{A1}-positive subject centrifuged at 2500*g* for 15 min (Lane 4), and serum from a PL^{A1}-positive subject (Lane 5).

short time (Lane 4). If the plasma is centrifuged sufficiently to eliminate platelet dust, that plasma has no effect on PL^{A1}-negative platelets (Lane 3).

Having found that with sufficient centrifugation neither the PL^{A1} epitope nor GPIIIa (data not shown) was detectable (at a detection sensitivity of 50–100 ng/lane) in soluble form in stored blood *in vitro*, we then turned our attention to looking for soluble GPIIIa *in vivo*. Figure 3A is an autoradiogram of an immunoblot of the plasma (2500*g* at 30 min) of a thrombocytopenic patient with acute PTP reacted with ^{125}I-labeled DEK-16 (Lane 2). By using this sensitive technique (capable of detecting <0.0003% of the GPIIIa of circulating platelets), we found no GPIIIa in the plasma of this PTP patient, nor was there any GPIIIa in a control fresh plasma (not agitated for 7 days) similarly centrifuged (Lane 3). Lane 1 represents a positive control, consisting of triton-solubilized whole platelets. Finding no soluble GPIIIa in plasma, we next looked for the antigen existing as platelet fragments in the 100,000*g* sediment. Panel B shows these experiments. The PTP patient's acute plasma from the 2500*g* centrifugation (Lane 1), the plasma from the 100,000*g* centrifugation (Lane 2), and the sediment of the 100,000*g* centrifugation (Lane 3) were run on immunoblot and developed with ^{125}I-DEK-16. As in Panel A, there was no GPIIIa in the plasma. In addition, there was no GPIIIa in the 100,000*g* sediment from the severely thrombocytopenic PTP patient on immunoblot. However, GPIIIa was present in the sediment of the supernatant of the 2500*g* plasma as well as the sediment obtained following 100,000*g* centrifugation of the 2500*g* supernatant of control plasma from subjects with normal platelet counts (data not shown). Lane 4 shows a positive control consisting of platelet lysate. Similar negative results were obtained after application of 1–2 ml of the PTP patient's centrifuged (100,000*g*) acute phase plasma to a 1-ml affinity column that had been coupled with DEK-16. This column was capable of affinity purifying GPIIIa from a platelet lysate (7). No GPIIIa could be eluted after overnight incubation of the column with the patient's acute phase plasma. Similar negative results were obtained with the acute plasmas of five patients with PTP, employing DEK-16 (two patients) as well as ^{125}I-BE (five patients) as the developing antibodies (data not shown).

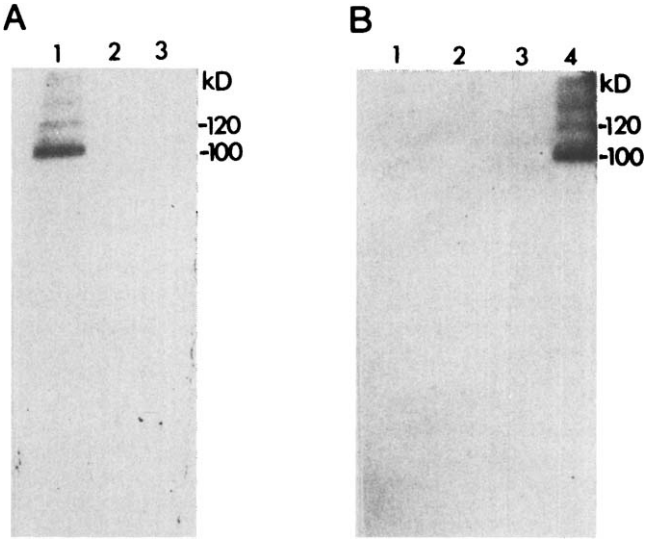

Figure 3. Autoradiogram of platelet and plasma specimens reacted with I^{125}-labeled DEK-16, a monoclonal antibody against GPIIIa. A shows the reactivity with 5×10^6 platelets (Lane 1), 100 μl of plasma (2500*g* at 30 min) from the patient BE with PTP (Lane 2), and 100 μl of plasma (2500*g* at 30 min) from a normal subject (Lane 3). B shows reactivity with the same antibody with platelets (Lane 4), plasma (2500*g* at 30 min) from the patient BE (Lane 1), plasma spun at 100,00*g* for 2 hr from the same patient (Lane 2), and a lysate of the 100,000*g* sediment of 1 ml of plasma in the bottom of the centrifugation tube solubilized in 200 μl of lysing buffer (Lane 3).

Discussion

The pathophysiology of PTP is enigmatic. It is clear that there is an interaction between antibodies and autologous platelets. Several hypotheses attempt to explain why these antibodies react with platelets which lack the antigen to which the antibody is made (13).

One hypothesis is that the antigen comes off the transfused PL^{A1}-positive platelets and somehow adheres to the recipient's platelets, thus converting the platelet phenotype from negative to positive. The antibody formed would then bind to the converted platelets and they would either be lysed by complement or cleared by the reticuloendothelial system. This hypothesis gained credence when data were published (4–6) showing that PL^{A1}-negative platelets could be converted to a PL^{A1}-positive phenotype by incubating them in plasma from stored blood drawn from a PL^{A1}-positive subject. By using an indirect assay, Kickler *et al.* (4) reported that the concentration of PL^{A1} epitope in the plasma of stored blood correlated with the duration of storage. The soluble PL^{A1} epitope, however, was not directly demonstrated. The soluble PL^{A1} was also not demonstrated by Dieleman *et al.* (6) who did not centrifuge their stored plasma at 100,000*g* prior to incubation with PL^{A1}-negative platelets. Warejcka *et al.* (5) reported that PL^{A1} platelets lost their PL^{A1} epitope in the absence of divalent cations, and that the epitope bound to PL^{A1}-negative platelets in the presence of divalent cations.

Our present data clearly show that the PL^{A1} epitope and the GPIIIa antigen are present in particulate form in stored blood and can be eliminated from the supernatants of platelet preparations by sufficient centrifugation. Furthermore, the presence or absence of divalent cations had no effect. With the use of a more specific assay than that employed by Kickler *et al.* (4), we have shown that 100,000*g* centrifuged plasma from stored blood from a PLA-1-positive donor is incapable

of converting PL^{A1}-negative platelets to a PLA-1-positive phenotype (within the detection sensitivity of our assay), irrespective of the duration of storage. It is possible that the data published by others reflect an artifact of platelet dust present in units of stored blood. These small platelet fragments resist centrifugation at 2500*g* in plasma. After mixing with washed PL^{A1} negative platelets, they cosediment with whole platelets during subsequent washings. When this material, consisting of PL^{A1}-negative platelets and PL^{A1}-positive platelet dust, is lysed in detergent, run on SDS-PAGE, and developed as an immunoblot, it produces a band of M_r 100,000 which reacts with an anti-PL^{A1} antibody.

Although our data suggest that transformation from a negative to a positive phenotype by a soluble antigen is an unlikely explanation for the pathophysiology of PTP, they do not exclude the possibility that insoluble fragments of PL^{A1}-positive platelets might bind to PL^{A1}-negative platelets, rendering them PL^{A1} positive. We consider this unlikely for the following reasons: (i) Insoluble platelet particles, passively introduced into a patient on one occasion should be rapidly cleared by their reticuloendothelial system. Indeed, the onset of thrombocytopenia classically occurs 1 week after the blood (platelet) transfusion and the duration of thrombocytopenia can be as long as two to three platelet survival intervals. (ii) We were unable to detect insoluble PL^{A1} dust in the 2500*g* plasma or 100,000*g* sediment of our severely thrombocytopenic PTP patient.

A second "immune complex" hypothesis proposes that on initial clearance of the PL^{A1}-positive platelets by anti-PL^{A1} antibody, fragmentation of these platelets results in the release of small amounts of PL^{A1}-positive material which form complexes with anti-PL^{A1} antibody. These complexes bind to PL^{A1}-negative platelets via their Fc receptors and result in accelerated clearance. On phagocytosis, the IgG-PL^{A1} complexes are released and bind to newly formed platelets (3). However, we were unable to detect soluble GPIIIa in the 100,000*g* plasma of five severely thrombocytopenic PTP patients or insoluble GPIIIa in the 2500*g* plasma or 100,000*g* sediment of a PTP patient. If this material had rapidly complexed with anti-PL^{A1} antibody, it might have been rapidly cleared and therefore not detected by our methodology.

A third hypothesis proposes that patients with this disorder develop auto- or alloimmune antibodies against other platelet antigens shared by PL^{A1}-negative and positive individuals. A recent report provides evidence for such an autoimmune antibody with specificity for a 120,000-dalton membrane glycoprotein (14).

PTP is a fascinating disorder. Hypotheses regarding the pathogenesis abound. Unfortunately, data supporting these hypotheses are either unavailable or unconfirmed. Our data argue against one appealing explanation for the disorder. It is our hope that by reporting negative data regarding one theory, we will accelerate the elucidation of the mechanism of this disorder.

This research was supported by Grant HL-13336-18 of the National Heart, Lung and Blood Institute.

The authors are indebted to Dr. Edward A. Amorosi of New York University Medical Center for generously providing the plasma exchange units of patient BE.

1. Zucker MB, Ley AB, Borrelli J, Mayer R, Firmat J. Thrombocytopenia with a circulating platelet agglutinin, platelet lysin and clot retraction inhibitor. Blood **14**:148, 1959.
2. van Loghem JJ Jr, Dorfmeijer H, Hart M, Schreuder F. Serological and genetical studies of a platelet antigen. Vox Sang **4**:161–69, 1959.
3. Shulman NR, Aster RH, Leitner A, Hiller MC. Immunoreactions involving platelets: V. Post-transfusion purpura due to a complement fixing antibody against a genetically controlled platelet antigen. A proposed mechanism for thrombocytopenia and its relevance in "autoimmunity." J Clin Invest **40**:1597–1620, 1961.
4. Kickler TS, Ness PM, Herman JH, Bell WR. Studies of the pathophysiology of posttransfusion purpura. Blood **68**:347–50, 1986.
5. Warejcka D, Janson M, Aster, RH. The PL^{A1} antigen, but not HLA antigens, can be acquired by platelets from plasma. Blood **66**:109a, 1985.
6. Dieleman LA, Brand A, Claas FHJ, van der Keur C, Witvliet M, Giphart MJ. Acquired Zw^a antigen on Zw^a negative platelets demonstrated by Western blotting. Br J Haematol **72**:539–542.
7. Kunicki TJ, Aster RH. Isolation and immunologic characterization of the human platelet alloantigen, PL^{A1}. Mol Immunol **16**:353–60, 1979.
8. Dancis A, Ehmann C, Ferziger R, Grima K, Karpatkin S. GPIIIa-related PL^{A1} antigen with different molecular weights: Studies in platelets, endothelial cells and megakaryocytes. Blood **71**:1056–61, 1988.
9. Lyman B, Rosenberg L, Karpatkin S. Biochemical and biophysical aspects of human platelet adhesion to collagen fibers. J Clin Invest **50**:1854–63, 1971.
10. Patscheke H. Shape and functional properties of human platelets washed with acid citrate. Hemostasis **10**:14, 1981.
11. Beardsley DS, Spiegel JE, Jacobs MM, Handin R, Lux SE. Platelet membrane glycoprotein IIIa contains target antigens that bind anti-platelet antibodies in immune thrombocytopenias. J Clin Invest **74**:1701–1707, 1984.
12. Johnson DA, Gautsch, JW, Sportsman, JR, Edler JH. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal Tech **1**:3, 1984.
13. Mueller-Eckhardt C. Annotation: Post-Transfusion Purpura. Br J Haematol **64**:419–24, 1986.
14. Stricker RB, Lewis BH, Corash L, Shulman MA. Post transfusion purpura associated with an autoantibody directed against a previously undefined platelet antigen. Blood **69**:1458–63, 1987.