Platelet to Leukocyte Adherence Phenomena Associated With Thrombocytopenia

By Philip R. Greipp and Harvey R. Gralnick

Two patients developed thrombocytopenia associated with an in vitro platelet to leukocyte adherence phenomenon (PLAP). In one patient, whole serum, intact plasma, and the IgG fraction of the serum could induce the PLAP of normal platelets in normal whole blood or buffy coat preparations. Passage of serum over a Sepharose column coupled with antihuman IgG resulted in the loss of all the PLAP activity. Activity could not be recovered from the whole serum or the IgG fraction of serum of the second patient; however, the PLAP was transferred to her newborn infant. In both patients, an unusual pulmonary illness accompanied the PLAP and thrombocytopenia.

A VARIETY of immunologically mediated thrombocytopenia(s) has been described. The diagnosis in vitro of an immune mediated thrombocytopenia has involved the detection of antibody binding to platelets, techniques involving platelet agglutination, or the measurement of antibody induced platelet injury. None of these techniques has implicated other circulating cells as playing a major role in the thrombocytopenia either through adherence or phagocytosis.

We would like to describe the phenomenon of platelet to leukocyte adherence in vitro associated with thrombocytopenia in vivo. The platelet to leukocyte adherence phenomenon (PLAP) was recognized in two patients. In both patients the PLAP developed in close correlation to an acute pulmonary distress syndrome.

The plasma, serum, and purified serum IgG from one patient could transfer the PLAP to normal blood, but in the second patient, the serum was nonreactive.

CASE REPORTS

Case I

G.T., a 61-yr-old female chronic alcoholic with hypersensitivity vasculitis, was admitted to the National Institutes of Health on December 31, 1970 for "weakness" of 3 wk duration. No symptoms of vasculitis were evident. Medications at the time of admission included cytoxan, 100 mg/day; folic acid, 5 mg/day; multivitamins; Darvon compound; and Librium.

Physical examination revealed a pale, acutely ill female. The only pertinent findings were a moderate number of retinal hemorrhages, microaneurysms of retinal vessels, and a soft liver edge 3 cm below the right costal margin.

Laboratory examination on admission revealed a hemoglobin of 3.9 g/100 ml with a hematocrit of 13.1% and a MCV of 109 cu μ, WBC 3100/cu mm with 68% segmented neutrophils, 4% bands, 7% lymphs, 19% monocytes, 1% eosinophils, 1% basophils, and 1% nucleated red blood cells per 100 WBC. Platelet count was 23,000/cu mm. Reticulocyte count was 0.9%. The peripheral blood smear made from EDTA anticoagulated blood showed minimal polymorphonuclear cell hyper-
segmentation; platelet morphology was normal. The only other abnormal laboratory results were 
a serum potassium of 1.9 mEq/liter, blood glucose of 122 mg/100 ml, and SGOT and SGPT of 
173 and 74 units, respectively. Urinalysis revealed a rare white cell and granular cast. I.E. cell 
preparations and antinuclear antibodies (ANA) were negative. A bone marrow biopsy and aspirate 
were hypocellular with megaloblastoid changes in both the white blood cell and red blood cell 
series. Megakaryocytes were decreased. The chest x-ray showed mild cardiomegaly.

Cytoxan was discontinued on admission. Initially transfusions of packed red cells were required 
to maintain her hemoglobin; however, during the second and third weeks of hospitalization, the 
WBC and hemoglobin spontaneously rose and stabilized at normal levels, and the platelet count 
rose to 174,000/cu mm. The bone marrow was then normoblastic and normocellular, and the 
megakaryocytes were normal in number and appearance. After recovery from the episode of 
marrow hypoplasia, the patient’s vasculitis was reevaluated.

Just before discharge from the hospital the patient complained of bilateral anterior chest pain 
with a nonproductive cough and tachypnea. Temperature was 38.3°C. Physical examination re-
vealed tachypnea and cyanosis without significant wheezes, rales, or rhonchi. A chest roentgeno-
gram revealed a right lower, middle, and upper lobe and a left lower lobe patchy diffuse inter-
stitial infiltrate. Multiple sputum cultures were negative, and cold agglutinins were negative. The 
patient’s condition became critical and therapy with pentamidine isothionate 200 mg/day intra-
muscularly was begun for suspected *Pneumocystis carinii* pneumonia. Shortly after the development 
of pulmonary symptoms the platelet count fell from 174,000 to 20,000/cu mm. The peripheral 
smear (prepared from EDTA anticoagulated blood) showed clumping of platelets and adherence 
and phagocytosis by neutrophils and monocytes (Fig. 1). This phenomenon continued on subse-
quent days (Fig. 2). Fingerstick platelet counts (done visually and by electronic particle count-
ers), as well as platelet estimates from the peripheral smear were performed to avoid problems 
with clumped or adherent platelets and phagocytosed platelets. A repeat bone marrow was done 
and showed normal numbers and morphology of the megakaryocytes. Coagulation and fibrinolysis 
studies were normal. However, high levels of fibrin(ogen) degradation products were found in the

Fig. 1. Peripheral blood smear from Case 1 which demonstrates multiple platelets adherent to 
a polymorphonuclear leukocyte. x 750.
Fig. 2. Correlation of PLAP score of whole blood, intact serum, purified IgG, and platelet count during course of the illness of Case I. Thrombocytopenia and pulmonary distress occurred simultaneously. Serotonin release with patient’s serum is negative before the PLAP occurs, but then becomes positive as platelet to leukocyte adherence and phagocytosis is observed.

urine at a time when the patient had become oliguric. This finding was associated with mild abnormalities in urinary sediment including a few hyaline and granular casts and one red cell cast. Blood urea nitrogen rose to 45 mg/100 ml; with the creatinine 1.5 mg/100 ml.

Following institution of steroid therapy (prednisone 60 mg/day) the patient’s pulmonary condition improved, the platelet count rose and the renal function improved. She was discharged with a platelet count of 250,000/cu mm, and the PLAP had decreased and then disappeared. The steroids were gradually tapered over a 3-mo period, and, on her last clinic visit 18 mo later, the patient had a normal hematocrit, hemoglobin, white blood cell count and platelet count, and clinically was much improved.

Case II

E.C., a 35-yr-old part-time nurse, was admitted to Georgetown University Hospital in August 1968, because of recurrent dyspneic episodes beginning 1 mo prior to admission. These episodes were characterized by a feeling of fullness in the chest and dyspnea which occurred both at rest and during exertion and lasted 10 to 15 min. The patient took no medications.

Physical examination and all laboratory studies were normal, except for the platelet count and the appearance of vacuolated neutrophils on the peripheral smear. They contained what appeared to be partially digested platelets. A platelet count done by the visual method was 53,000/cu mm. Clumped platelets were noted in the chamber on several repeat platelet counts, and blood smears showed the PLAP (Fig. 3).

Chest roentgenogram showed a prominent pulmonary artery on the left with normal vasculature on the right, and a lung scan showed multiple small scalloped areas of hypoventilation in the peripheral areas bilaterally.

Because of the possibility of pulmonary emboli, the patient was given heparin for 14 days. The patient experienced no dyspneic attacks, and the lung scan improved. The patient was seen 2 yr later at the time of the delivery of her second child, a normal healthy male infant. Although the infant was normal, EDTA anticoagulated blood did demonstrate PLAP. PLAP and platelet phagocytosis were still present on smears of EDTA anticoagulated blood of the mother, although her platelet count was normal.
MATERIALS AND METHODS

PLAP

Blood was collected into EDTA anticoagulated tubes (Becton-Dickinson) from which smears were made within 30 min with a modified Wright's stain. Ten normals (five males and five females) served as controls. Their platelet counts ranged from 180,000 to 397,000/cu mm. Peripheral blood smears from EDTA anticoagulated blood were examined from three patients with autoimmune thrombocytopenic purpura and ten patients with systemic lupus erythematosus. PLAP scores were determined by carefully scanning the feathered edge in an area appropriate for the study of red cell morphology. To assess the degree of PLAP, each of 100 segmented neutrophils and monocytes was scored as follows: 0—no platelets adherent or phagocytosed; 1—one single platelet adherent or phagocytosed; 2—two single platelets adherent or phagocytosed; 3—three single platelets adherent or phagocytosed; 4—four or more single platelets adherent or phagocytosed. The PLAP score was expressed as the sum of the points for the 100 cells. PLAP was looked for in these samples kept at both 25°C and 37°C. Blood collected by the fingerstick technique was smeared directly, stained, and scored as above.

Platelet Counts

Platelet counts were performed in duplicate by phase microscopy and by electronic particle counting (Coulter Electronics, Hialeah, Fla.). Platelet count estimates were utilized to compare with the observations of phase counting as follows: the same area of the peripheral blood smear was used as in determination of the PLAP score. The number of platelets present in each of ten high-power fields (HPF) was determined in triplicate. Platelet number per HPF was reported as the average number of platelets seen in a single HPF.
In Vitro Transfer of the PLAP Factor

Experiments were performed with plasma or serum or the IgG fraction of serum added to fresh compatible blood or Buffy coat and incubated at 37°C. Plasma separation was performed after blood was collected by venipuncture and placed into glass tubes containing 5.0 U of heparin/ml whole blood or 0.5 mg EDTA/ml whole blood. Centrifugation was at 5000 g for 15 min. Serum was separated in the same manner from blood allowed to clot in glass tubes for 2 hr at 37°C. Plasma or serum was utilized within 4 hr of separation. Appropriate dilutions were made with phosphate (0.01 M) buffered saline (0.14 M) pH 7.4. In transfer experiments, normal sera or plasma and buffered saline served as controls. PLAP was determined on blood samples at various intervals from 0-90 min incubation at 25°C or 37°C. Scores reported were obtained at 30 min, since incubation beyond this time resulted in no further PLAP.

Buffy coat preparations were obtained from EDTA anticoagulated blood centrifuged at 2000 g for 15 min and washed thrice in phosphate-buffered saline pH 7.4. EDTA had been added to the buffer at a final concentration of 0.5 mg/ml to prevent platelet clumping during centrifugation. The Buffy coat was resuspended before use to its original volume in buffer.

Purification of IgG

Serum was chromatographed on DEAE-cellulose.4 The IgG fraction was concentrated to the original serum volume and incubated with washed Buffy coat from normals and scored. An IgG fraction from normal serum served as a control.

IgG Absorption

Serum from Case I and normal sera were passed over a column containing Sepharose (Pharmacia, Piscataway, N.J.) covalently linked to monospecific antiserum to human IgG. The effluent was concentrated to a protein concentration equal to the serum prior to absorption. IgG could not be detected in the effluents on immunoelectrophoresis against antihuman IgG (Mel Par Laboratories, Fairfax, Va.). PLAP scores were then performed using the IgG absorbed serum as above.

Immunofluorescent Study

One drop of fluorescein isothiocyanate, labeled monospecific antihuman IgG, antihuman IgM or antihuman β-thromboglobulin (Mel Par Laboratories, Fairfax, Va.) diluted 1:4 in phosphate-buffered saline pH 7.4, was added to 1 drop of washed Buffy coat. The slides were prepared and coded by one observer, then evaluated for fluorescence independently by two other observers. Results were tabulated and then the code was broken.

Serotonin Release

Serotonin release was performed on normal platelets incubated with heated sera from the patient (Case I) and normals as previously described.5

RESULTS

PLAP Scores

PLAP scores from normal EDTA anticoagulated or fingerstick blood fell into a normal range of 1 to 4; normal saline, serum, or plasma added to normal EDTA anticoagulated control whole blood or Buffy coat gave the same result. (Tables 1 and 2).

In Case I, the PLAP scores were normal during the initial episode of thrombocytopenia (associated with a hypoplastic marrow). The elevated PLAP scores and apparent platelet phagocytosis seen on EDTA anticoagulated blood smears and phase microscopy were closely associated with the discovery of the second episode of thrombocytopenia (Fig. 2). EDTA anticoagulated blood smears 2 days prior to the development of thrombocytopenia showed no PLAP.
As the second episode of thrombocytopenia improved following the institution of steroid therapy, the PLAP score progressively fell and finally disappeared. The peak score was 380 (possible 400). The PLAP was observed only in EDTA anticoagulated blood. It was maximal at 30 min and similar degrees of PLAP were observed at 25°C and 37°C. The PLAP scores with heparin anticoagulated or fingerstick blood were normal. During recovery of the thrombocytopenia, the PLAP progressively fell, and the serum was inactive in inducing the PLAP.

In Case II, the PLAP varied from 122–156. This patient’s PLAP was observed at both 25°C and 37°C, and the PLAP score was maximal 30 min after collection of EDTA anticoagulated blood. The PLAP score was normal in blood collected in heparin and in smears made directly from fingerstick blood. Although all tests of plasma, serum, and purified IgG from Case II gave negative results, EDTA anticoagulated blood from her newborn infant demonstrated PLAP and phagocytosis by segmented neutrophils and monocytes. The PLAP score of the infant’s EDTA anticoagulated blood was 104. The PLAP scores were normal in ten patients with systemic lupus erythematosus, three patients with idiopathic thrombocytopenia purpura, and 25 patients with miscellaneous disorders and thrombocytopenia selected at random.

**In Vitro Transfer of the PLAP Factor**

In transfer experiments, the serum, plasma, and purified IgG of Case I induced the PLAP of normal EDTA anticoagulated whole blood or buffy coat preparations prepared from EDTA anticoagulated whole blood (Table 1). The PLAP for undiluted serum incubated with whole blood in equal parts, was 42; and for serum: buffer (dilution 1:1), was 16; phagocytosis was observed at both dilutions. The PLAP became normal at a serum:buffer dilution of 1:2. IgG prepared from this serum added to an equal volume of buffy coat gave a PLAP
of 232 (Table 1), while anti-IgG absorbed serum, normal human serum, or isotonic saline gave a normal PLAP.

**Immunofluorescent Studies**

Normal buffy coat preparations incubated with fluorescent antihuman IgG, IgM, or α,β-C showed no platelet fluorescence. Fluorescent antihuman IgG demonstrated fluorescence of the platelets in the buffy coats of Cases I and II. The observation of brightly fluorescent droplets in several leukocytes was striking. This fluorescence corresponded to cytoplasmic vacuoles. No platelet or leukocyte fluorescence was observed with antihuman IgM and anti-α,β-C.

**Serotonin Release**

In the serotonin release experiments serum samples from Case I converted from negative to positive (Fig. 2) concomitant with the development of the PLAP.

**DISCUSSION**

PLAP or platelet satellitism is one of several causes of spurious thrombocytopenia. It is considered a curiosity seen only with EDTA anticoagulated blood smears. On these smears, segmented neutrophils adhere to, and often phagocytose autologous platelets. In previous reports this phenomena has been characterized by (1) absence of true thrombocytopenia, (2) the absence of PLAP on fingerstick blood smears, and (3) adherence of platelets to granulocytes. Thus, most reporters believe that this is merely an in vitro phenomenon of little clinical significance. Variable results have been obtained with transfer experiments.

Our observations extend some of these earlier characterizations, since thrombocytopenia has been observed in both our patients with PLAP. There is recent evidence in the literature that thrombocytopenia can result from the interaction of phagocytic cells and platelets and a new in vitro test system utilizes platelet leukocyte adherence to demonstrate antibody to platelets. Thus, the discovery of in vivo thrombocytopenia with PLAP demanded further investigation; the possibility of an antibody-mediated reaction was explored.

The cells involved in platelet adherence and phagocytosis were segmented neutrophils and monocytes. The interaction was essentially unaltered by temperature; it occurred at 37°C, as well as at 25°C, as previously reported. Both serum and its IgG fraction in patient I were able to transfer PLAP activity to normal blood and buffy coat. The removal of activity by passage of the serum over a column containing monospecific anti-IgG antibody bound to Sepharose supported the role of IgG in the PLAP in Case I. Identification of IgG on the platelet surface by fluorescent antihuman IgG and brilliant fluorescence in the leukocyte vacuoles supported the concept that in both patients the IgG sensitized platelets first adhered to, and then were engulfed by leukocytes.

Platelet phagocytosis in PLAP has been confirmed by electron microscopy by Reisman et al. and by Kjeldsberg and Swanson. Ultrastructural studies were not performed on our patient’s blood; however, phase microscopy observations confirmed platelet adherence and subsequent phagocytosis in EDTA anticoagu-
lated blood. These observations suggested that the final common pathway of thrombocytopenia in our patients could have been phagocytosis in vivo by neutrophils, monocytes, and possibly splenic macrophages.

An unknown heat-labile EDTA-dependent factor enhanced platelet leukocyte adherence in Verp and Karpatkin's patients. The role of complement in PLAP, therefore, remains unclear. No fluorescence of the platelet surface was observed with fluorescent anti-\( \beta_1 \)C in our patients, and the washeduffy coat and purified IgG carried out the reaction (PLAP) without complement. In addition, heated serum caused serotonin release, even though several components of complement should have been inactivated. Complement may not be necessary for the adherence of IgG-coated red cells to phagocytic cells, as receptors for IgG have been demonstrated on the surface of neutrophils, monocytes, and have been postulated on splenic macrophage. Platelets might adhere in a similar manner when IgG is present on their surface. Why PLAP should require EDTA anticoagulant is uncertain. Thus, the exact mechanism of the reaction is unclear, although it appears in some cases to be mediated by an IgG immunoglobulin.

The significance of the pulmonary symptoms in these two patients is also uncertain. Perhaps there is an in vivo interaction between platelets and the leukocytes in the pulmonary circulation; alternatively, the PLAP and the pulmonary problem may have been coincidentally related in these patients.

Whether PLAP is a clue to an underlying systemic problem, or simply a manifestation of a platelet antibody in some patients, it is clear that the occurrence of thrombocytopenia in these cases of PLAP emphasizes the need for fuller understanding of platelet immunophagocytosis. Studies using leukocytes and platelets and other test cells could help clarify such interactions and with it some of the mechanisms of thrombocytopenia. Difficulty in demonstrating platelet antibodies in patients with platelet satellitism or PLAP suggest that sensitive tests using leukocytes as proposed by Handin and Stossel or the procedure of Verp and Karpatkin are clearly needed.

We conclude that antibody mediated PLAP may represent not only a cause of spurious thrombocytopenia, but that it can be associated with thrombocytopenia in vivo.

ACKNOWLEDGMENT

The authors wish to thank Dr. Richard J. Hirschman for performing the serotonin release experiments and Ms. Dawn Gilbert and Mrs. Marty Auman for excellent secretarial assistance.

REFERENCES

Platelet to leukocyte adherence phenomena associated with thrombocytopenia

PR Greipp and HR Gralnick