Isolation of Human Platelet Membrane Microparticles From Plasma and Serum

By James N. George, Linda L. Thoi, Linda M. McManus, and Terry A. Reimann

Methods have been developed to isolate human platelet membrane fragments from plasma and serum. Rabbit antibody produced against the human platelet membrane glycoprotein complex, IIb/IIIa, was utilized in an immunoelectrophoretic assay to evaluate the amount of this antigen in various microparticle preparations. The serum concentration of platelet microparticles was more than tenfold greater than that observed for plasma (65 μg/ml versus 4.4 μg/ml, respectively). Ultrastructural evaluation of either plasma or serum-derived microparticles disclosed a variety of membrane fragments and membrane-bound vesicles with occasional fragments of red blood cells, white blood cells, and platelets. In contrast, microparticle preparations derived from isolated washed platelets after thrombin stimulation contained a heterogeneous array of membrane fragments, vesicles, and granules but no identifiable red cell, white cell, or platelet fragments. Thus, these studies demonstrate that normal human plasma and serum contain platelet membrane fragments that are produced during cell activation. If a similar loss of platelet membranes occurs in vivo following reversible platelet activation, it is possible that the resulting membrane modifications may be of importance in both the structural and functional changes that develop during platelet senescence.

MINUTE PARTICLES in normal human plasma and serum, termed "platelet dust," were first described by Wolf, who found that this material contained most of the platelet-related coagulant activity of whole plasma and serum.1 Crawford2 extended these observations and concluded that these microparticles were formed by in vivo fracture of membrane buds from extended platelet pseudopods. To define further human platelet derived microparticles, we developed an immunoelectrophoretic assay for a human platelet membrane glycoprotein complex. This assay was used to characterize platelet membrane microparticles in vitro and to estimate the amounts of these microparticles in plasma and serum of normal human subjects.

MATERIALS AND METHODS

Antibody Preparation

Four New Zealand white rabbits were immunized with washed human platelets emulsified with an equal volume of Freund's incomplete adjuvant. Each rabbit was immunized subcutaneously with 200 μg protein3 3 times at 2-wk intervals and blood was drawn 1 wk after the final injection. IgG was isolated from serum by ammonium sulfate precipitation and DEAE-cellulose column chromatography4 and then dialyzed against Tris-glycine buffer (0.10 M glycine, 0.04 M Tris-HCl, pH 8.7).3 Crossed immunoelectrophoretic (CIE) analysis of both microparticles isolated from plasma (see below) and whole platelets using unadsorbed anti-whole platelet IgG yielded multiple immunoprecipitin arcs. The most prominent arc in both samples was the platelet membrane glycoprotein (GP) complex IIb/IIIa, identified by its absence from the platelets of a patient with Glanzmann's thrombasthenia5 (patient reported in references 6 and 7). Using this unadsorbed antibody, an arc of identity formed between this IIb/IIIa immunoprecipitate of whole platelet and microparticle samples when they were analyzed together by tandem crossed immunoelectrophoresis.6

The antibody solutions were then adsorbed first with the cryoprecipitate fraction of normal plasma and subsequently with platelets from a patient with Glanzmann's thrombasthenia.6,7 Cryoprecipitate was prepared from 50 ml of blood anticoagulated with 10 ml of acid citrate dextrose (ACD)9 containing 300 ng/ml PGE, (kindly provided by Dr. John E. Pike, Upjohn, Kalamazoo, Mich.) and 6 mM dibutyryl cyclic AMP (db-cAMP). Plasma was isolated by centrifugation at 35,000 g for 60 min, followed by incubation at 4°C overnight. The cryoprecipitate was isolated by centrifugation at 10,000 g for 10 min at 4°C and washed once with cold saline. The cryoprecipitate was dissolved in 30 ml of the antibody solution at 37°C for 60 min, incubated overnight at 4°C, then resolated and separated from the antibody as described above. Next, the antibody (30 ml) was adsorbed with the particulate fraction of sonicated thrombasthenic platelets. Platelets from 50 ml of blood were washed, sonicated, and the pellet of insoluble material was isolated by centrifugation at 35,000 g for 60 min. The pellet was resuspended in the antibody solution and incubated at 37°C for 60 min, followed by incubation at 4°C overnight. The platelet pellet was then resolated from the antibody by centrifugation as described above. The final protein concentration was 23.0 mg/ml in the antibody preparation used in these experiments. The antibody was stored at 70°C in 5-ml aliquots and after thawing were kept at 4°C with 0.01% sodium azide.

Platelet Microparticle Preparation

For plasma microparticle samples, 30 ml of blood was drawn from subjects, with informed consent, through a 19-gauge needle of a "miniset" vein infusion set (Travenol Laboratories, Inc., Deerfield, Ill.) into a plastic syringe containing 5 ml of ACD9 with PGE, (300 ng/ml), after discarding the first 10 drops. Only samples that were obtained by an easy venipuncture with quickly flowing blood were used. The sample was immediately placed on ice and centrifuged at 5000 g for 6 min at 4°C within 15 min. Ten milliliters of plasma were
removed, diluted with 30 ml of cold Ringer's citrate dextrose (RCD) PGE1, pH 6.5, 300 mosmole to reduce the density of the medium and centrifuged at 35,000 g for 20 min at 4°C. The pellet, 5-10 mm in diameter, was resuspended with a Pasteur pipette in a small volume of RCD-PGE₁ and transferred to a 0.6 ml tube and centrifuged again at 35,000 g for 20 min. The washed pellet was resuspended in 40 μl of Tris-glycine buffer by sonication and solubilized by the addition of 10 μl of 10% (vol/vol) Triton X-100 at 30°C for 60 min. Then the mixture was centrifuged at 35,000 g for 20 min and the supernatant fluid used promptly for immunoelectrophoresis.

Serum was obtained from 10 ml of whole blood clotted by mixing with glass beads at 37°C for 60 min and then centrifuged at 5000 g for 6 min. Microparticles from 1 ml of serum were isolated and solubilized as described above.

To prepare blood depleted of platelets, blood was centrifuged 5 times at 400 g for 3 min. After each centrifugation, the top half of the supernatant was removed from the volume restored with RCD-PGE₁-BSA (0.35%). White cells were counted in a hemocytometer, and the differential count was performed on a Wright's-stained smear. CaCl₂ (5 mM) was added to the blood-buffer mixture, which was then clotted with thrombin (1 U/ml) for 60 min at 37°C and subsequently centrifuged at 10,000 g for 10 min (homogeneous human α-thrombin, 2600 U/mg, was a gift of Dr. John W. Fenton, New York State Department of Health, Albany, N.Y.). Microparticles were isolated from this "serum" as described above.

Platelets were separated from contaminating white cells and red cells in anticoagulated whole blood by centrifugation at 160 g for 20 min. The top half of the platelet-rich plasma was removed and the platelets were washed 3 times in RCD-PGE₁-BSA at 1000 g for 15 min and resuspended (10⁶/ml) in Tyrode's buffer containing 0.35% BSA and 5 mM CaCl₂. The ratio of white cells to red cells to platelets was determined with a hemocytometer and phase-contrast microscope. In these preparations, there was less than one white cell and one red cell/6000 platelets. After aggregation with 2 U/ml thrombin for 10 min at 37°C, the platelet suspension was then centrifuged at 10,000 g for 10 min and microparticles were isolated from the supernatant fluid as described above.

Whole platelet lysates for standards in the quantitative immunoelectrophoretic assay were prepared from washed platelets obtained from ACD-PGE₁-BSA, anticoagulated blood by centrifugation at 120 g for 20 min and washing 3 times in RCD-PGE₁-BSA. The washed platelets were resuspended in RCD-EDTA buffer containing protease inhibitors,10 total protein determined, and frozen in aliquots at −70°C. For each immunoelectrophoresis assay, an aliquot of whole platelets was thawed and solubilized with Triton X-100 in parallel with the microparticle samples.

**Immunoelectrophoresis**

Immunoelectrophoresis (IEP) was performed by the method of Hagen et al. and Axelsen et al. using 1% (w/v) agarose containing 1% (v/v) Triton X-100 in a thickness of 1.5 mm on glass plates. For crossed immunoelectrophoresis (CIE), approximately 100 μg of platelet protein were separated in the first dimension (10 V/cm, 1 hr, 12°C). In the second dimension, these proteins were electrophoresed into agarose containing approximately 400 μg/sq cm of antibody (1.5 V/cm, 18 hr, 12°C) (FBE-3000 immunoelectrophoresis apparatus, Pharmacia, Uppsala, Sweden; 2095 circulating water bath, Forma Scientific, Marietta, Ohio). Quantitative single dimension rocket IEP was performed by the method of Laurell as described by Axelsen et al. using agarose with 200 μg/sq cm of antibody (1.5 V/cm, 18 hr, 12°C). All samples were applied to the wells in a 10-μl final volume. After electrophoresis, the agarose plates were pressed, washed, dried, and stained with Coomassie blue. The 125I-labeled monoclonal antibody (Tab) against the platelet membrane glycoprotein complex IIb/IIIa was kindly supplied by Rodger McEver, M.D., University of Texas Health Science Center, San Antonio, and was used in an intermediate gel of CIE as described by Hagen et al. The 125I-Tab was visualized by autoradiography of the stained CIE plate for 1 hr at room temperature using Kodak XAR-5 film and Dupont "Cronex Lightning Plus" intensifying screens.

Platelet factor 4 was assayed by Shirley P. Levine, M.D., University of Texas Health Science Center, San Antonio. Platelet counts were performed on an electronic particle counter (Thrombocounter-C, Coulter Electronics, Inc., Hialeah, Fla).

**Electron Microscopy**

Microparticle samples were processed for electron microscopic evaluation by resuspending the washed pellet of microparticles in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C for 1 hr. After centrifugation at 35,000 g for 20 min at 4°C, the fixative was removed and the fixed pellet was washed in 0.1 M cacodylate buffer, pH 7.2, and subsequently postfixed in 1% osmium tetroxide. After further washing and dehydration, the pellet was embedded in Epon-araldite. Ultrathin sections were obtained and counter-stained with aqueous uranyl acetate and lead citrate before viewing in a Philips 301 electron microscope at an operating voltage of 60 kV.

**RESULTS**

**Antibody Specificity**

Rabbit anti-human platelet IgG samples were adsorbed with cryoprecipitate and thrombasthenic platelets. After adsorption, a single major immunoprecipitin arc formed with crossed immunoelectrophoresis (CIE) using solubilized whole platelets as the antigen (Fig. 1). The specificity of the adsorbed antiserum for the platelet membrane GP IIb/IIIa complex was confirmed using a radioiodinated monoclonal antibody (Tab) directed against GP IIb/IIIa in an interme
diate gel with two-dimensional crossed immunoelectrophoresis (Fig. 1). Autoradiography demonstrated that the $^{125}$I-Tab was superimposed on the major immunoprecipitin arc. Although other minor immunoprecipitates were visible by Coomassie blue staining after CIE of the adsorbed antibody against solubilized whole platelets, these did not interfere with interpretation of the quantitative single dimension rocket immunoprecipitates.

**Platelet Microparticle Preparation and Characterization**

To determine the centrifugal force required to obtain platelet-free plasma, platelet counts and PF4 concentrations were measured (Table 1). No platelets and negligible PF4 were present in plasma obtained by centrifugation of whole blood at forces equivalent to the product of 20,000 g $\times$ min. Therefore 5000 g for 6 min (equivalent to 30,000 g $\times$ min) was used as the standard force for obtaining plasma and serum for the isolation of microparticles. The platelet microparticles were quantified by immunoelectrophoresis with the adsorbed antibody using serial dilutions of whole platelet samples on each agarose plate as standards, and the results were recorded as the equivalent value of whole platelet protein.

Additional evidence for the platelet origin of the microparticles was provided by two separate experiments. No measurable immunoprecipitates were observed when microparticle samples were prepared from serum obtained after clotting platelet-depleted blood (Fig. 2a). In spite of the absence of the platelet antigen, a visible pellet was obtained with the serum indicating the occurrence of nonplatelet microparticles. In contrast, immunoprecipitates were formed between the adsorbed antibody and the supernatant fluid obtained after thrombin-induced aggregation of washed platelets that were free of red cells and white cells (Fig. 2b).

![Fig. 2. Quantitative immunoelectrophoresis of platelet microparticles (MP).](image)

The plasma concentration of platelet microparticles in normal subjects was $4.4 \pm 3.2$ (SD) $\mu$g/ml ($n = 20$, 12 individuals). PF4 concentration was measured in 14
of these 20 plasma samples and equalled 9.6 ± 4.2 (SD) ng/ml, within the normal range for this laboratory. Among these normal samples there was no correlation between the microparticle concentration and the PF4 concentration. When plasma was collected from whole blood by the standard centrifugation (5000 g for 6 min), most of the microparticles (1.5 µg/ml) were recovered by the standard pelleting force used for the diluted plasma (35,000 g for 20 min) compared with a fourfold greater force (150,000 g for 20 min) (2.1 µg/ml, mean of 2 experiments). No experiments were performed with an increased centrifugal force of the whole blood to obtain plasma because the normal concentration of plasma microparticles was near the lower limit of the IEP accuracy.

The concentration of platelet microparticles was greater in serum than in plasma: 65.2 ± 29.1 (SD) µg/ml (n = 6, p < 0.001) using the standard centrifugal forces, equal to those used for plasma. Increasing the force of the initial centrifugation of the clotted whole blood decreased the serum platelet microparticle concentration, but some material was not sedimented by centrifugation at 20,000 g for 10 min (Table 2). Therefore, some platelet microparticles are present in serum in a very minute form, such as small membrane vesicles.

Electron microscopic analysis of the microparticle pellets obtained from plasma and serum demonstrated a very heterogeneous array of subcellular components. Although the majority of the structures observed from these microparticle preparations was composed of membrane fragments and membrane-bound amorphous material, fragments of red blood cells, white cells, and platelets were occasionally identified. In order to assess the ultrastructural morphology of only the platelet-derived components of microparticle preparations, microparticles obtained from thrombin-aggregated isolated platelets were evaluated. These preparations were composed of an acellular array of membranes and membrane-bound vesicles of varying size, shape, and density, as well as some non-membrane-enclosed flocculent material (Fig. 3). These structures were similar to the acellular components of the microparticle preparations derived from plasma and serum. Although none of the structural elements present in Fig. 3 can be morphologically identified as coming specifically from platelets, the fact that they were produced from a platelet suspension that was free of white cells and red cells (see Materials and Methods) indicates their platelet origin.

**DISCUSSION**

The material obtained by high speed centrifugation of diluted plasma and serum contains a heterogeneous mixture of cellular debris. This may be related to the production of membrane fragments, which has been described in a variety of cells. Red cell fragments result from membrane budding and may be related to the mechanism of cell senescence. Spicules of plasma membrane can also be lost due to reversible change of red cell shape in sickle cell anemia. Loss of plasma membrane blebs has also been observed in other cells in tissue culture. In patients with malignant diseases, tumor cell antigens, possibly representing plasma membrane fragments, may be present in a high concentration in cell-free plasma. The identification and analysis of circulating cell membrane products may provide an easily accessible opportunity for the study of normal cell kinetics, accelerated cell destruction, and the characteristics of abnormal cells.

The platelet-related coagulant activity of cell-free plasma and serum is particulate and sedimentable. This material has been postulated to be derived from fragmentation of the platelet plasma membrane. We developed an immunoelectrophoretic assay with an antibody against the platelet membrane glycoprotein complex Iib/IIia to specifically identify and quantitate membrane microparticles in plasma and serum. The immunoprecipitin arcs of GP Iib/IIia in the microparticle samples were compared to the arcs from serial concentrations of whole platelets, and then the microparticle concentrations were expressed as the equivalent value of whole platelet protein. This estimate may not accurately reflect platelet microparticle total protein, since the ratio of GP Iib/IIia to total protein may be different in microparticles and whole platelets. Also, microparticle composition may be different in different conditions, for example, in plasma versus serum. This problem cannot be resolved until multiple antigens can be measured in both microparticles and whole platelets to assess their relative concentration, or until platelet microparticles can be isolated.
Fig. 3. Ultrastructural appearance of a microparticle preparation obtained from the supernatant of a washed platelet suspension (free of white cells and red cells) after stimulation by thrombin as described in Materials and Methods. At low magnification (A), note the heterogeneous structures of varying size, shape, and density. Of particular interest, dense structures resembling elongated platelet dense bodies were uniformly but randomly distributed throughout these preparations. At higher magnifications (B-D), greater detail of the various components of the microparticle preparation is presented. Occasional multilamellar membranes were observed (B); however, these structures were not routinely associated with any of the other more commonly observed constituents, such as the dense structures or other membrane-enclosed granules. Flocculent material with no apparent enclosing membrane was always observed (C); no regular pattern of distribution of this material with other structures was ascertained. Infrequently, structures resembling cell organelles such as mitochondria, were present in these samples (center, D). Bars represent 0.5 μ.

Platelet microparticles were found in all normal plasma samples and were present in a tenfold greater concentration in serum. However, neither the structural origin nor the mechanism of development of the platelet microparticles is known. Our immunoassay is specific for the platelet membrane glycoprotein complex IIb/IIIa, but these glycoproteins are present in intracellular granule membranes as well as the surface plasma membrane. The morphology of the microparticles isolated from thrombin-aggregated washed platelets, free of white cells and red cells, demonstrated that much of the material was membrane-bound. Some structures appeared similar to membrane-enclosed amorphous cytoplasm; other structures were smaller, empty membrane vesicles; and some particles resembled intact platelet granules and dense bodies. The most dense material was frequently present in an elongated form, similar to the dense bodies from activated platelets described by White and Skaer. Regardless of the subcellular origin of these microparticles, their existence must be considered in
the preparation of plasma and serum samples for assays that could be affected by platelet or membrane vesicle activities.

An important question is whether or not these microparticles exist in vivo or are only formed as a result of inadvertant platelet stimulation during blood collection. The development of assays for platelet-secreted proteins in plasma has emphasized the great care that is required for obtaining plasma samples without in vitro platelet activation.\textsuperscript{15,29} Although we did not specifically compare different methods of blood collection, anticoagulation, and the temperature of the sample preparation, it is known that these variables affect the plasma concentration of platelet-secreted proteins.\textsuperscript{13,29} Therefore, in the present study, in contrast to the earlier observations,\textsuperscript{11,23,30,31} we utilized procedures designed to minimize platelet activation and secretion during blood collection. The low concentration of PF4 in the plasma samples indicated that a minimal degree of in vitro platelet secretion had occurred. Further study will be required to determine if the optimal conditions for obtaining plasma for microparticle analysis are the same as for measuring platelet-secreted proteins, and the same for samples for normal subjects and patients with platelet abnormalities.

Microparticle formation could be related to in vivo platelet activation, including secretion and reversible aggregation. Studies in rabbits have demonstrated the continual loss of membrane fragments from circulating platelets.\textsuperscript{32,33} This process can be retarded by the infusion of agents that inhibit platelet function and can be accelerated by the infusion of thrombin to produce intravascular coagulation.\textsuperscript{33,34} This loss of plasma membrane may account for the smaller size and decreased hemostatic effectiveness of older rabbit platelets.\textsuperscript{35} Platelet membrane fragmentation may be accelerated when platelets are exposed to turbulent circulation and high shear stress\textsuperscript{30} or are damaged in vivo by autoantibodies\textsuperscript{30,31} These membrane fragments caused by in vivo platelet thrombi may be analogous to the platelet membrane microparticles produced in clotting whole blood in the present study as well as in earlier experiments.\textsuperscript{37} Further analysis of platelet membrane fragmentation may provide better insight into the mechanism of normal platelet senescence and abnormalities with accelerated platelet destruction.

ACKNOWLEDGMENT

We thank Daniel Staskawicz for his expert technical assistance and Judi Skinner for her preparation of this manuscript.

REFERENCES


of spectrin-free spicules on reoxygenation of sickled erythrocytes. Nature 295:613, 1982
Isolation of human platelet membrane microparticles from plasma and serum

JN George, LL Thoi, LM McManus and TA Reimann