ABSENCE OF PLATELET MEMBRANE GLYCOPROTEINS IIb/IIIa FROM MONOCYTES

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Glycoproteins IIb/IIIa form a Ca^{2+}-dependent complex that functions as the fibrinogen receptor on human platelets (1). Several reports have indicated that this fibrinogen receptor is also expressed on the surface of human monocytes (2–4). Of three monoclonal antibodies directed against platelet IIb/IIIa, two were reported to give a positive reaction with monocytes as well (4). Other investigators reported that, in patients with Glanzmann's thrombasthenia, a bleeding disorder characterized by the absence of or marked reduction in IIb/IIIa on platelets, both glycoproteins were also missing in monocytes (3). It has also been shown that monocytes bind fibrin (5, 6), which would be consistent with the presence of a fibrinogen receptor on their surface.

The presence of IIb/IIIa on monocytes would offer a good opportunity to study the biosynthesis and molecular biology of these glycoproteins and hence to determine the genetic defect in bleeding disorders like Glanzmann's thrombasthenia. Monocytes are accessible in large numbers and are easily kept in culture, in contrast to megakaryocytes, the platelet precursor cells.

Despite much evidence, however, the presence of IIb/IIIa on mononuclear phagocytes is still not fully established. Monocytes that are isolated from blood are often contaminated with adherent platelets unless scrupulous precautions are taken (7–9). Thorough washing may remove most platelets but fragments of platelet membrane can remain attached to the monocyte surface by an interaction mechanism that is still unclear. We have recently worked out a method for the large scale preparation of highly purified monocytes from donor blood using centrifugal elutriation. Since our preparations were virtually free of platelets, we decided to reinvestigate IIb/IIIa on mononuclear phagocytes. Using two different techniques based on mono- and polyclonal antibodies, as well as immunoelectron microscopy, we found no evidence for the presence of these glycoproteins on monocytes and monocyte-derived macrophages.

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Materials and Methods

Platelets. Human blood platelets were isolated, within 20 h after donation, from citrated blood (Central Laboratory, Blood Transfusion Service, Swiss Red Cross, Bern) washed, and labeled as described by Steiner et al. (10).

Monocytes. Pure monocyte populations were obtained essentially according to the method of Leijh et al. (11) with minor modifications. Briefly, citrated blood from healthy donors was centrifuged at room temperature on Lympho-paque (Nyegaard Co., Oslo, Norway) containing 0.6 mM sodium citrate. Cells from the interphase were washed once at 10°C with phosphate-buffered saline containing 1 U/ml heparin, then washed three times in 4% pasteurized plasma protein solution (PPL SRK 4%; Swiss Red Cross) containing 0.5 mM EDTA. Monocytes were then isolated from this population by centrifugal elutriation using a JE6 elutriator rotor operated by a refrigerated J6-M centrifuge (Beckman Instruments, Inc., Palo Alto, CA). The elutriation medium consisted of Ca^{++} and Mg^{++}–free phosphate-buffered saline, pH 7.4, containing 0.01% EDTA and 1% bovine serum albumin. The flow rate was kept constant (18 ml/min), the temperature was 10°C, and the rotor speed was reduced stepwise from 3,130 to 2,100 rpm. Monocytes were collected in two 45-ml fractions, at each speed (2,430, 2,330, and 2,230 rpm) and kept in ice. The cells were washed once (250 *g* for 10 min) in Dulbecco's modified Eagle's medium (DME)[1] (Gibco, Basle) and either used directly or cultured for 4 d in DME containing 3% pooled group AB human serum (Swiss Red Cross).

The purity of the monocyte preparations used in these experiments was >90%, judged morphologically after Giemsa staining and by peroxidase and esterase histochemistry, as well as by fluorescence-activated cell sorter analysis using anti-Leu-M3 antibody (Becton, Dickinson & Co., Mountain View, CA).

Surface Labeling and Immunoprecipitation. Monocytes (5×10^7) were labeled by the periodate/[^{3}H]$NaBH_4$ method as adapted for platelets by Steiner et al. (10). After washing, the monocytes were solubilized in 400 μl of 0.15 M NaCl, 0.005 M EDTA, 0.5% Nonidet P-40, 0.05 M Tris-HCl, pH 7.4, followed by centrifugation at 100,000 *g* for 30 min. The supernatant was used for immunoprecipitation and for two-dimensional gel electrophoresis.

Immunoprecipitation was performed overnight at 4°C by incubating 100 μl of the solubilized ^{3}H-labeled monocyte preparation with 50 μl of rabbit antiplatelet IgG prepared according to Hagen et al. (12). Goat anti-rabbit Immunobeads (4 mg; Bio-Rad Laboratories, Richmond, CA) were added and the mixture incubated for a further 4 h. The Immunobeads were washed three times with 0.15 M NaCl, 0.005 M EDTA, 0.5 Nonidet P-40, 0.05 M Tris-HCl, pH 7.4 buffer; then 100 μl of 0.125 M Tris-HCl, 2% sodium dodecyl sulfate (SDS), pH 6.8 were added and the mixture was heated to 100°C for 2 min. The supernatant was directly used for SDS–polyacrylamide gel electrophoresis.

Gel Electrophoresis. One- and two-dimensional gel electrophoresis of immunoprecipitates and radiolabeled platelet and monocyte preparations were carried out; gels were prepared for fluorography and exposed to Kodak X-Omat SO-282 film as previously described (13).

Crossed Immunoelectrophoresis. Supernatants from solubilized platelet and monocyte preparations (80 μg protein) were separated by crossed immunoelectrophoresis against rabbit antiplatelet antibodies essentially as described by Hagen et al. (12). The first dimension consisted of 1% agarose in 1% Triton X-100, 0.038 M Tris, 0.1 M glycine, pH 8.7. Electrophoresis was carried out at 10 V/cm for 1 h at 10°C. The second dimension was performed on Gel Bond film (FMC Corp., Chicago, IL) in 1% agarose at 2 V/cm for 18 h at 10°C. An intermediate gel contained ^{125}I-labeled P4 (0.1 μg/cm^2) monoclonal antibody to glycoproteins IIb/IIIa (14), while the main gel contained polyclonal rabbit antiplatelet antibodies (11 μg/cm^2) either purchased from Dakopatts, Copenhagen, Denmark or prepared by repeated injection of washed human platelets into New Zealand rabbits. The gels were washed, stained with Coomassie Blue, and dried. The

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate.

dried gels were exposed to Kodak X-Omat SO-282 film between two Dupont Cronex Lightning Plus intensifying screens (DuPont Co., Wilmington, DE).

Labeling of Monoclonal Antibody. The P4 monoclonal antibody (100 μg) was labeled with ^{125}I (1 mCi) using the chloramine T method (15). The labeled mixture was applied to a Sephadex G25 column, and the excluded peak was collected, protein content and radioactivity determined, and aliquots stored at −70°C.

Immunocytochemical Procedures. Platelets and monocytes separated from human blood by the Ficoll-Hypaque method were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer for 1 h at 37°C, and were subsequently washed and processed as described by Stenberg et al. (16). They were then treated with mouse monoclonal antibody to platelet IIb/IIIa (T10; reference 17) followed by goat anti-mouse antibodies linked to colloidal gold (Janssen Pharmaceuticals, Beerse, Belgium) at a dilution of 1:20. Cells processed with colloidal gold immunoconjugates were postfixed in 1% OsO_4, exposed to uranyl acetate, and then processed for transmission electron microscopy.

Results

Surface Labeling and Immunoprecipitation. Monocytes were isolated by elutriation and surface labeled by a variant of the periodate/[^{3}H]$NaBH_4$ technique (10). After solubilization and centrifugation, the supernatant was either separated by two-dimensional polyacrylamide gel electrophoresis (Fig. 1) or used for immunoprecipitation with rabbit antiplatelet antibodies and then analyzed by SDS–polyacrylamide gel electrophoresis (Fig. 2). Surface labeled platelets were used in parallel as a positive control (Fig. 2).

The two-dimensional separations showed completely different patterns for platelets and monocytes. No spot in the monocyte fluorogram corresponded to the normal positions of platelet IIbα, IIIa, or IIbβ (compare Fig. 1, *a* and *b*). A normal pattern of platelet surface glycoproteins was obtained after electropho-

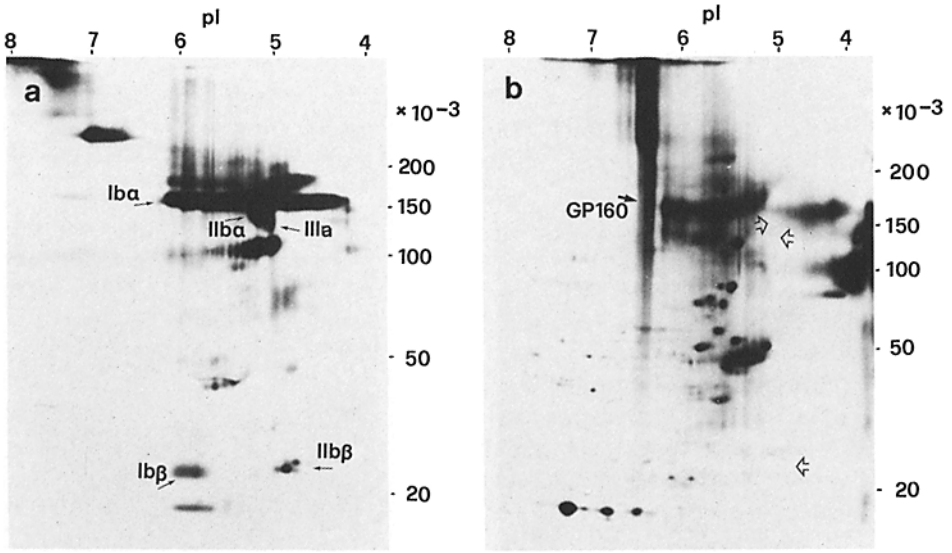

FIGURE 1. Fluorograms of two-dimensional polyacrylamide gel electrophoresis separations of cells surface-labeled by the periodate/[^{3}H]$NaBH_4$ technique. (*a*) Human blood platelets; the glycoproteins that have been reported to occur in monocytes are indicated. (*b*) Human monocytes; the position of the major glycoproteins is indicated (*GP160*) as are the positions where the platelet IIb/IIIa complex would be expected (arrows).

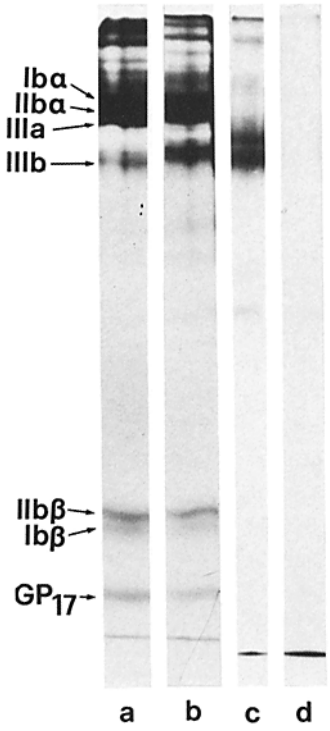

FIGURE 2. Fluorogram of one-dimensional SDS–polyacrylamide gel electrophoresis of cells surface-labeled by the periodate/[^{3}H]$NaBH_4$ technique, and of immunoprecipitates with rabbit antiplatelet antibodies. (*a*) Human blood platelets. (*b*) Immunoprecipitate platelets. (*c*) Human monocytes. (*d*) Immunoprecipitate monocytes. The original fluorogram of *d* showed faint bands corresponding to the main bands in *c*.

retic analysis of immunoprecipitates of solubilized platelet samples (Fig. 2, *a* and *b*). No bands corresponding to the major platelet glycoproteins, however, could be identified on electrophoresis of the monocyte samples subjected to the same immunoprecipitation procedure (Fig. 2 *d*). Very faint bands were found on the latter gel corresponding to some monocyte glycoproteins (Fig. 2, *c* and *d*). They are probably due to nonspecific precipitation since they were also present in control samples that were immunoprecipitated using preimmunization rabbit serum (not shown).

Crossed Immunoelectrophoresis. Solubilized samples of freshly prepared monocytes and platelets and of monocyte-derived macrophages obtained after 4 d in culture were used. Platelet preparations were solubilized either in the presence of Ca^{2+} (5 mM) or EDTA (5 mM) because of the known Ca^{2+} dependence of the IIb/IIIa complex (1).

All samples were then subjected to crossed immunoelectrophoresis against rabbit anti–whole platelet antibodies using ^{125}I-labeled monoclonal anti IIb/IIIa antibody (P4; reference 14) in an intermediate gel. As shown by Fig. 3, in the platelet sample a heavy precipitation arc characteristic of IIb/IIIa was obtained (Fig. 3 *a*), which was strongly labeled by the monoclonal antibody in the autoradiograms (Fig. 3 *b*). No other arc was revealed by this antibody. The gels obtained

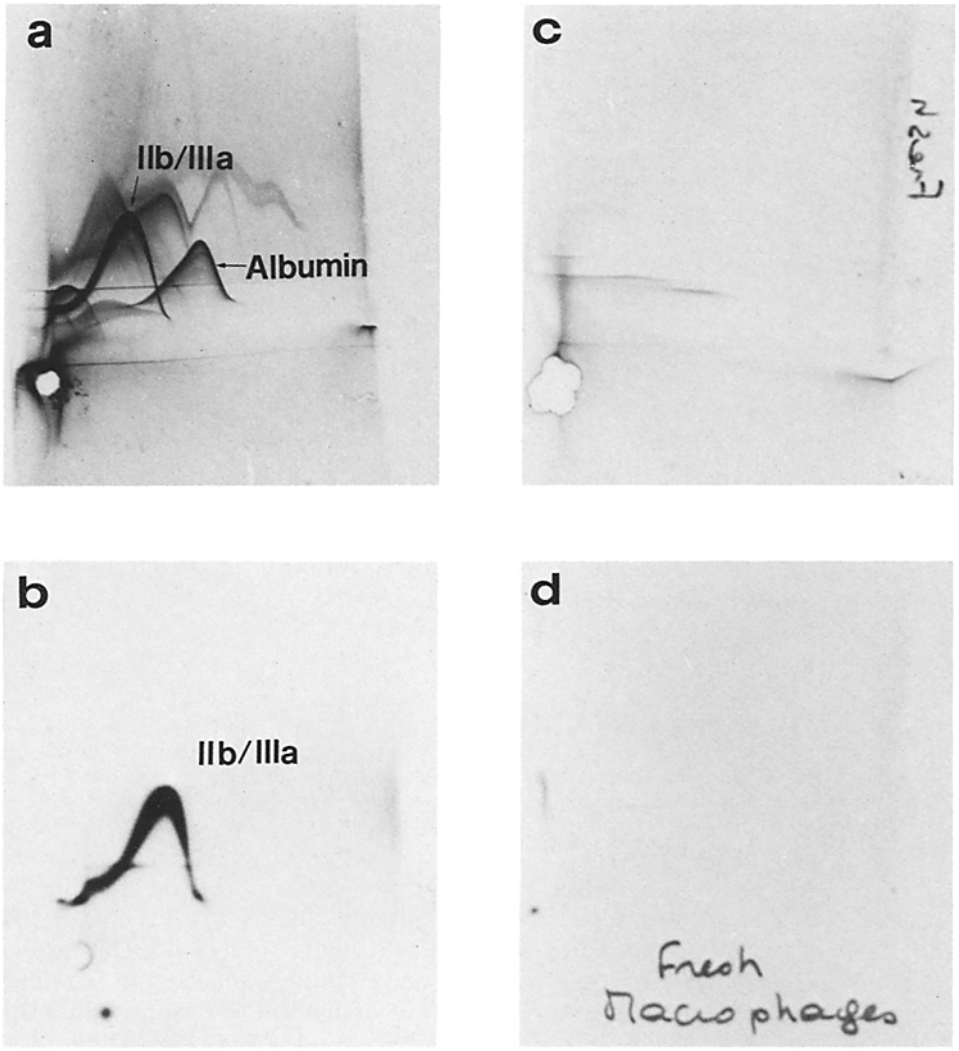

FIGURE 3. Crossed immunoelectrophoresis studies with solubilized platelets and monocytes and a monoclonal antibody to platelet glycoproteins IIb/IIIa. (*a*) Coomassie Blue–stained gel of 80 μg of platelet protein solubilized in 1% Triton X-100 in the absence of EDTA, with ^{125}I-labeled anti-IIb/IIIa monoclonal antibody (P4) in the intermediate gel. The upper gel contained polyclonal rabbit anti-human platelet antibodies. (*b*) Autoradiogram of gel shown in *a*. (*c*) Coomassie Blue–stained gel of 80 μg of protein from fresh monocytes solubilized in 1% Triton X-100 in the absence of EDTA, with ^{125}I-labeled anti-IIb/IIIa monoclonal antibody (P4) in the intermediate gel. The upper gel contained polyclonal rabbit anti-human platelet antibodies.

with the mononuclear phagocyte samples showed much less staining. No precipitation arc could be identified from fresh monocytes (Fig. 3*c*), which were completely negative on prolonged autoradiography (Fig. 3*d*). Coomassie Blue staining of the gels obtained from monocytes cultured for 4 d revealed one major arc corresponding to albumin and some minor ones that probably also represented serum proteins taken up from the culture medium (not shown). Only one,

very faint arc was detectable on autoradiography (not shown), corresponding to the albumin precipitate.

Immunocytochemical Demonstration of IIb/IIIa. Mixtures of monocytes and platelets were fixed, treated with monoclonal antibody to IIb/IIIa (T10; reference 17), treated with goat anti–mouse IgG coupled to gold particles, and then processed for electron microscopy. Figs. 4, 5, and 6 show typical examples of platelets and monocytes in close proximity. While the platelet membranes show regular tagging with gold, the monocyte membranes are virtually free of label. In the rare instances where gold particles were present near monocyte surfaces, closer examination revealed their association with a fragment of membrane, presumably derived from a platelet.

Discussion

Two different immunochemical techniques were applied to the identification of glycoproteins IIb/IIIa in platelets, monocytes, and monocyte-derived macrophages from human blood. Both detected the glycoproteins in platelets but not in mononuclear phagocyte preparations. Compatible results were obtained by immunochemistry using a monoclonal antibody against the platelet glycoproteins (revealed by a gold-labeled second antibody), which bound heavily to the platelet but not to the monocyte surface.

The presence of a platelet-like fibrinogen receptor on mononuclear phagocytes has been a much debated question over the past few years. Most reports to date have suggested that IIb/IIIa are indeed common to both cells. Our results now show that this is most probably not the case. The inherent problem in studies of this nature is obtaining platelet-free monocytes to analyze. Platelets adhere to the monocytes and, unless special precautions are taken, most monocytes carry a few platelets on their surface. This problem has been discussed in detail by Pawlowsky et al. (9) and also addressed by other authors (7, 8). Whole platelets associated with monocytes are easily seen but membrane fragments of platelets disrupted by the preparation method may escape detection. Even sophisticated treatments to dissociate the platelet-monocyte microaggregates are only partial solutions.

Evidence for the presence of IIb/IIIa on monocytes falls into three categories: detection on monocytes themselves (2, 4); the failure to detect these glycoproteins on monocytes from patients with Glanzmann's thrombasthenia (3) (a bleeding disorder characterized by the absence or marked reduction of IIb/IIIa on platelets); and detection on cell lines of a monocytic nature (2, 4).

The absence of IIb/IIIa on monocytes from Glanzmann's thrombasthenia (3) may then be attributed either to their absence from the platelets or to the lower tendency of such platelets to aggregate and be activated (18). Treatment of monocytes with EDTA has been described as an effective way of removing adhering platelets (5, 8). It is known that platelets treated with EDTA no longer aggregate in response to a variety of stimuli (19), and this has been shown (1) to be related to the dissociation of the IIb/IIIa complex, which is Ca^{2+}-dependent and which acts as the fibrinogen receptor on the platelet (1). Glanzmann's thrombasthenia platelets also fail to aggregate to the same stimuli (18). Therefore, it would not be surprising if thrombasthenic platelets also do not stick to

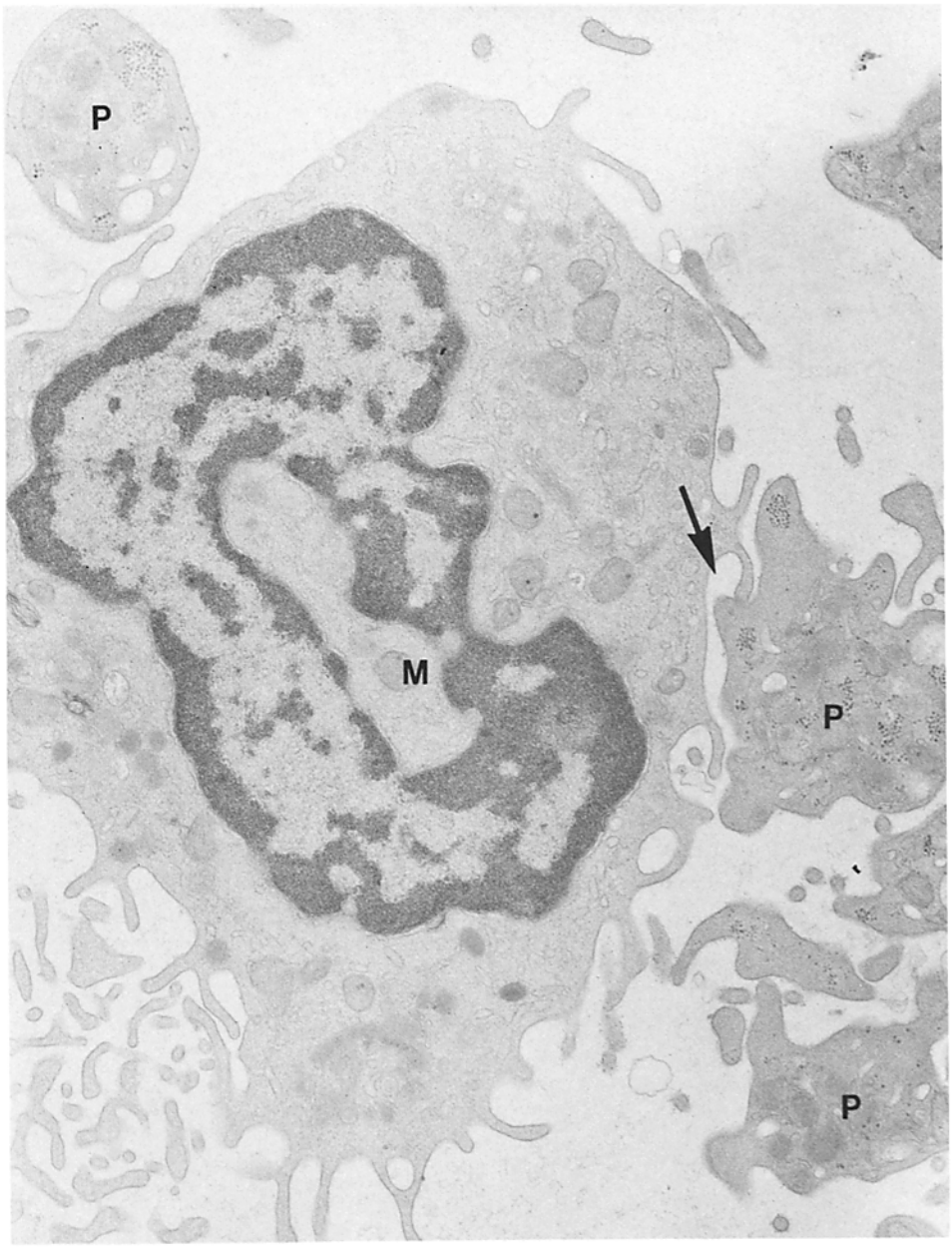

FIGURE 4. Transmission electron micrograph of monocyte (*M*) and platelets (*P*) from human blood. Notice the frequent clumping of platelets next to the monocyte. In addition, these cells were processed for the immunocytochemical localization of the monoclonal antibody for glycoproteins IIb/IIIa using the immunogold probe GAM-5. It is difficult to appreciate at this magnification, but the plasma membranes of the platelets are heavily labeled, in a patchy manner, along their entire extent, while the plasma membrane of the monocyte is free of gold particles. The area marked by the arrow can be seen at higher magnification in Fig. 6. × 17,000.

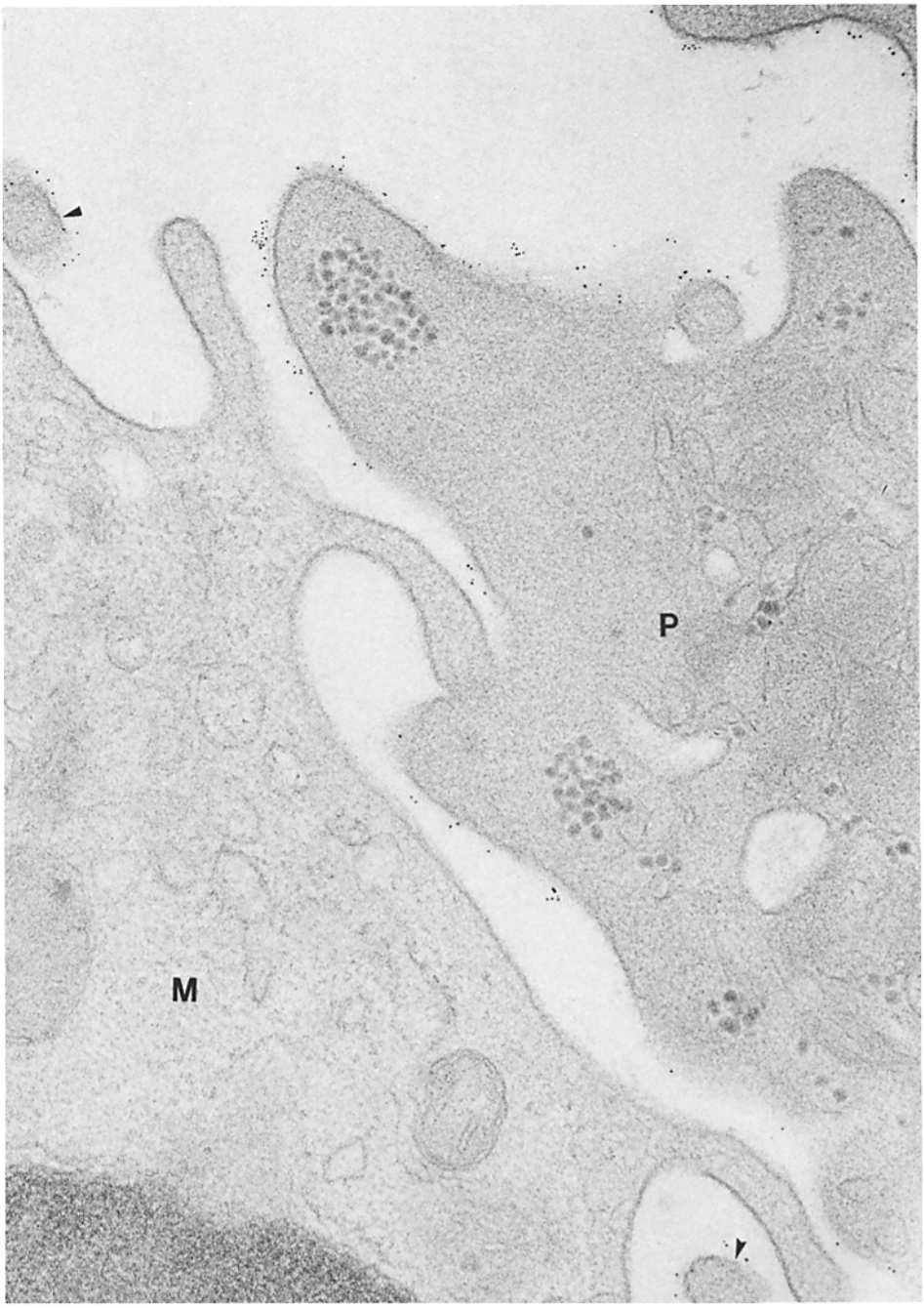

FIGURE 5. A higher- magnification view of a portion of the cells seen in Fig. 5., more clearly illustrating the presence of gold particles along the platelet (*P*) plasma membrane and their absence from the monocyte (*M*) plasma membrane. Small portions of platelet membranes (arrowheads) have been sectioned and are also labeled with gold particles. × 66,000.

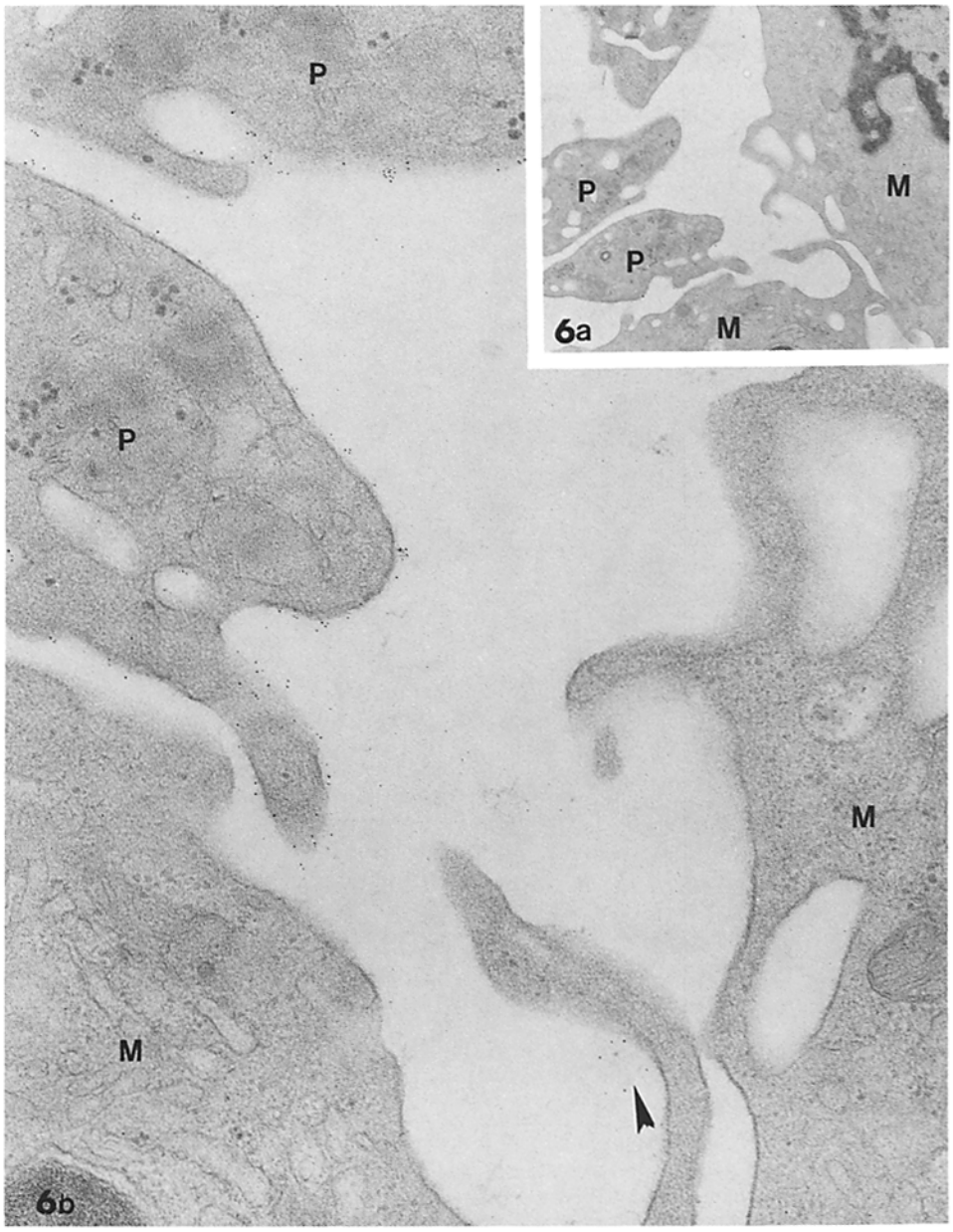

FIGURE 6. A low-magnification view of two monocytes (*M*) and adjacent platelets (*P*). Note the presence of immunogold label for IIb/IIIa on platelet membrane and its absence from the monocyte membranes. The small circular patch of label on the left (arrowhead) is not on the monocyte membrane and is probably a grazing section through the tip of platelet pseudopod. (*a*) × 11,000; (*b*) × 55,000.

monocytes. However, we cannot yet distinguish whether platelet membrane fragments are absent on thrombasthenic monocytes due to the platelets not sticking or if we are unable to detect these fragments because they do not contain IIb/IIIa.

Another line of evidence in favor of the presence of IIb/IIIa on mononuclear phagocytes is their detection on several cell lines (4, 20). The use of cell lines avoids the problem of contamination by platelets, but does not reveal the properties of mononuclear phagocytes, since these lines are derived from leukemia or lymphoma patients and may be incompletely or incorrectly differentiated. The HEL line, for example, expresses both erythrocyte and megakaryocyte membrane markers, including IIb/IIIa (21). Even in cell lines such as HL-60, the expression of IIb and IIIa is controversial, having been found by two groups (2, 4) but not by a third (20).

One of the principal arguments (4) in favor of the presence of IIb/IIIa on monocytes, based on studies with cell lines, was that two out of three monoclonal antibodies, which react with IIb/IIIa on platelets, selectively bind to both monocytes and cell lines of monocytic character. The remaining monoclonal is, however, capable of precipitating the IIb/IIIa complex. The authors suggested that the IIb/IIIa complex might have a different conformation on the monocyte than in platelets. Since little is known of the epitopes or avidity of these monoclonals, a simpler explanation cannot be excluded, namely that this monoclonal has a lower avidity and reacts poorly with the lower density of IIb/IIIa found on cell lines or on the fragments of platelets associated with monocytes.

Our studies failed to detect glycoproteins IIb/IIIa in preparations of 2×10^7 mononuclear cells, using techniques that would detect the glycoproteins in 10^6 platelets. This shows that the monocyte preparations contained <5% of platelets on a per cell basis or 0.4% on a surface membrane basis. The latter figure assumes a diameter of 10 μm for a monocyte (22) and 0.96 and 3.72 μm for the axial and equatorial diameters, respectively, of a discoid platelet (23). Our inability to detect platelets in these preparations by either microscopic or biochemical techniques indicates that centrifugal elutriation yields monocytes with minimal contamination by platelets. It is difficult from published work to estimate the levels of IIb/IIIa found on monocyte preparations, but these seem to be comparable to the amounts present on type II Glanzmann's thrombasthenia platelets (24), which are ~10% of those present on normal platelets. Since the techniques we have used would detect lower amounts than this, it seems likely that the positive results of other authors were due to undetected contamination of the monocytes by platelet fragments.

Summary

Two-dimensional gel electrophoresis, immunoprecipitation, and crossed immunoelectrophoresis were used in the investigation of glycoproteins IIb/IIIa in platelets, monocytes, and monocyte-derived macrophages from human blood. All techniques detected the glycoproteins in platelets but not in the mononuclear phagocytes. Similar results were obtained by immunochemistry using a monoclonal antibody against the platelet glycoproteins IIb/IIIa (revealed by a gold-labeled second antibody) which bound heavily to the platelet but not to the

monocyte surface. The biochemical techniques used for the analysis of mononuclear phagocytes would have reliably detected the level of glycoproteins IIb/IIIa contributed by a 5% contamination with platelets, calculated on a per cell basis.

We conclude that human monocytes and monocyte-derived macrophages lack glycoproteins IIb/IIIa. Our results further indicate that centrifugal elutriation yields monocyte preparations with minimal contamination by platelets. It seems likely that the positive results obtained by other authors were due to the presence of platelets or fragments on the monocytes.

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