

Immunocytochemical Localization of Fibrinogen, Platelet Factor 4, and Beta Thromboglobulin in Thin Frozen Sections of Human Blood Platelets

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ABSTRACT Affinity-purified monospecific antibodies against human fibrinogen and the platelet-specific proteins platelet factor 4 and beta thromboglobulin were used to localize these antigens in thin and ultrathin frozen sections of mildly fixed, washed human blood platelets. By immunofluorescent double-labeling experiments the distribution of fibrinogen was compared to that of platelet factor 4 and beta thromboglobulin. All three antigens occurred in virtually all platelets and showed an identical, dotlike distribution. For immunoelectron microscopy we used protein A-colloidal gold on ultrathin frozen sections to visualize the specific reaction indirectly. The staining for platelet factor 4, beta thromboglobulin, and fibrinogen localized exclusively over α -granules of washed platelets. Within the granules, platelet factor 4 was localized preferentially in the electron dense, α -granule nucleoid, whereas fibrinogen was more predominant in the electron-lucent granule periphery. Beta thromboglobulin localization did not show a preferential intragranular distribution.

INTRODUCTION

Blood platelets play a crucial role in the hemostatic process in that they arrest bleeding (1) and support blood vessel repair (2) and wound healing (3). Platelets can be considered as anucleate secretory cells that discharge their secretory products upon specific stimuli, such as thrombin or collagen (4). Unlike many other

secretory cells, platelets do not undergo a recovery phase (4).

The localization of the secretory substances within the platelet is important for understanding the platelet secretory process. Using morphologic criteria three possible secretory granule classes have been recognized: alpha granules, dense granules, and lysosome-like granules (5, 6).

With biochemical cell fractionation techniques fibrinogen, platelet factor 4, and beta thromboglobulin were demonstrated in platelet fractions containing alpha granules (3, 7-9). Morphologic evidence for these findings is provided by immunofluorescence studies (10-13). This technique, however, has a poor resolving power. Attempts to localize fibrinogen with the electron microscope failed (14). Yet, more detailed morphological information on the intracellular distribution of these substances is necessary in order to know whether the localization is restricted to α -granules and also whether they are stored within the same granule or whether subpopulations of granules are present with different contents.

In this study we have used immunoelectron microscopy to investigate the subcellular distribution of fibrinogen, platelet factor 4, and beta thromboglobulin. We preferred to work with thin frozen sections (15, 16) because they combine optimal accessibility of intracellular antigens to the immunoreagents with a good preservation of cellular ultrastructure. This technique has been successfully used for the demonstration of Factor VIII-related antigen (FVIII R:Ag)¹ in blood platelets by means of fluorescent antibodies (12, 17).

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¹ Abbreviations used in this paper: FVIII R:Ag, Factor VIII-related antigen; PAG, protein A-colloidal gold; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

METHODS

Methods were performed at room temperature, unless stated otherwise.

Chemicals. Theophyllin (1-3-dimethylxanthine), prostaglandin E_1 , 3,3'-diaminobenzidine · 4 HCl, benzamidine, and diisopropylfluorophosphate were obtained from Sigma Chemical Co. (St. Louis, MO). Polyethyleneglycol (6,000 mol wt) and methylcellulose (Tylose MH-300) were from Fluka AG (Buchs, Switzerland). Protein A (*Staphylococcus aureus*), Sepharose 6B, and CNBr-CL4B were from Pharmacia Fine Chemicals (Uppsala, Sweden). Imidazole was from J. T. Baker Chemical Co. (Phillipsburg, NJ). Alanine was from Koch Light Lab. Ltd. (Colnbrook, Bucks, England). Tetramethylrhodamine isothiocyanate was from B.B.L. Microbiology Systems, Becton, Dickinson & Co. (Cockeysville, MD); fluorescein isothiocyanate and Freund's complete adjuvant were from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, CA); glutaraldehyde was from Ladd Research Industries, Inc. (Burlington, VT); paraformaldehyde and ferritin were from Polysciences, Inc. (Warrington, PA); horseradish peroxidase-labeled goat anti-rabbit and rabbit anti-goat antibodies and chondroitin-6-sulfate from shark cartilage was from Miles Yeda Ltd. (Rehovot, Israel) the other chemicals were analytical grade from Merck & Co., Inc. (Darmstadt, FRG).

Antibodies. Antifibrinogen from goat and rabbit was prepared as follows: fibrinogen was purified as described by Jakobsen and Kierulf (18) and Polson and Ruiz-Bravo (19) with minor modifications. The fibrinogen preparation showed as two closely spaced bands after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (20) without reduction and as three bands, the characteristic $A\alpha$, $B\beta$, and γ chains (21-23), with reduction.

Antibodies against purified fibrinogen were raised in rabbits by four weekly injections of 25 μ g of fibrinogen and in goats by six weekly injections of 50 μ g fibrinogen, all in Freund's complete adjuvant. 1 wk after the last injection the animals were bled, and antifibrinogen IgG was affinity purified by adsorption to fibrinogen coupled to cyanogen bromide-activated agarose beads (CNBr-CL4B) according to the supplier's recipe (5 ml gel volume, 4 mg fibrinogen/ml agarose). The antibodies were eluted with 1 M KCNS and dialyzed for 16 h against phosphate-buffered saline (PBS; 10 mM phosphate, 150 mM NaCl; pH 7.4, 260 mosmol/kg). Monospecificity of these antifibrinogen preparations were first tested by immunodiffusion. Both, the goat and rabbit antibodies, gave one precipitation line against whole plasma, whereas no line against serum was observed. For a second specificity test we used a modified SDS-gel immunoperoxidase technique (24): fibrinogen and washed platelets (see platelet preparation below) were boiled for 5 min in standard reduction mixture (20) and 50- μ g samples were submitted to slab gel electrophoresis in a 7-15% gradient gel (SDS-PAGE) until the dye front had migrated for 5 cm into the separation gel. Subsequently the two lanes were cut out together to form a block of $\sim 50 \times 25 \times 4$ cm, of which 40 μ m thick longitudinal cryosections (59×25 mm) were cut; after fixation in ethanol/acetic acid (85:14, vol/vol) and washing in PBS the gel sections were first incubated with specific antibodies. Immunoreactive bands in the gels were visualized through a second incubation with anti-first-step antibodies conjugated to horseradish peroxidase, resulting in a brown reaction product when mixed with diaminobenzidine and H_2O_2 . On gels of both washed platelets and purified fibrinogen, the immunoreaction was restricted to three bands after incubation with goat or rabbit antifibrinogen.

The bands corresponded to the three fibrinogen chains after Coomassie Blue staining. This indicated that the antifibrinogens were specific for fibrinogen and that no other platelet proteins were detected.

Platelet factor 4 and beta thromboglobulin were isolated and characterized as described before (25, 26). Purity of both antigens was >99% as determined by SDS-PAGE and radioimmunoassay (27, 28). Antibodies to the two proteins were raised in rabbits and affinity purified by adsorption to insolubilized, pure antigens and eluted by glycine-HCl, pH 3. The specificity of antiplatelet factor 4 and anti- β -thromboglobulin γ -globulin was tested with the 'Western blot' technique on samples electrophoresed after reduction in linear gradient gels of 3-30% polyacrylamide with 2.4% cross-linking. The Western blots were prepared according to Towbin et al. (29) with minor modifications, using nitrocellulose sheets (BA 85, 0.45 μ m Schleicher & Schüll, FRG) and electrophoresis for 30 min at 10 V/cm.

The antibody against β -thromboglobulin stained the only Coomassie Blue band visible when up to 20 μ g of β -thromboglobulin was applied to the gradient. In the reduced platelet sample two closely spaced bands were visualized. The upper band is probably identical to the basic platelet protein, which was related to β -thromboglobulin (30). The lower band corresponded to β -thromboglobulin. Antiplatelet factor 4 did not cross-react with β -thromboglobulin and anti- β -thromboglobulin did not stain purified platelet factor 4. Purified platelet factor 4 was visible as a single Coomassie Blue band on the gradient when up to 20 μ g was applied to a gradient gel. Antiplatelet factor 4 globulin stained this band and faintly stained a band of the same apparent molecular weight as purified platelet factor 4, in platelets. Diffuse staining of various other areas of the lane containing reduced platelet proteins was consistently observed. This staining is probably caused by the extreme stickiness of platelet factor 4, which binds to other platelet proteins, and this binding is not disrupted by reduction and treatment with SDS and urea. Absorption of the antibody with platelet factor 4 solubilized by adding chondroitin-6-sulfate (shark) in a concentration 1:1 abolished the staining of the purified platelet factor 4, the platelet factor 4 band in platelets, as well as the more diffuse staining. Absorption with chondroitin sulfate alone did not abolish the staining.

Immunomarkers. For light microscopy goat antifibrinogen was conjugated directly to fluorescein. The rabbit antibodies were visualized with goat anti-rabbit IgG conjugated to rhodamine (31, 32). For electron microscopy we only used the specific rabbit antibodies, which were marked indirectly by ferritin conjugated to goat anti-rabbit IgG (33) or by a protein A-colloidal gold (PAG) complex. PAG was prepared as described before (34). Gold particles with an average size of 5 nm were prepared by reduction of gold chloride with white phosphorus and complexed with protein A according to Roth et al. (35).

Platelet preparation. Platelets were freshly fixed (a) or after washing (b). (a) Blood was collected by venipuncture and mixed with 10 vol of fixative. After 15-30 min a platelet pellet was prepared by differential centrifugation. (b) For washed platelet preparations blood (9 ml) was collected and mixed with 1 ml of a solution containing 20 mM theophyllin, 220 mM EDTA, and 1 mM prostaglandin E_1 , prepared immediately before use. Blood and inhibitor solution were mixed by inverting the tube three times. 10 ml of the blood mixture was diluted with a mixture of 9 ml Krebs-Ringer buffer (4 mM KCl, 107 mM NaCl, 20 mM $NaHCO_3$, 2 mM Na_2SO_4 ; pH 6.5, 260 mosmol/kg) and 1 ml of the inhibitor mixture. Platelet-rich plasma was prepared by centrifuga-

tion at 135 *g* for 10 min, pipetted off and transferred to a plastic tube in which the platelets were pelleted at 350 *g* for 10 min. Next the platelets were washed three times with 10 ml of the Krebs-Ringer buffer by repeated resuspension and centrifugation at 500 *g* for 10 min. Finally the pellet was resuspended in fixative and pelleted after 15–30 min. 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) was used as fixative for light microscopy studies. For electron microscopy 0.1% glutaraldehyde was added to the fixative. Pellets of fixed platelets were mixed with 10 ml of a 2.3 M sucrose solution in PBS. After 10 min the platelets were pelleted (500 *g*, 10 min) the supernatant sucrose solution was discarded and the pellets were mixed with small volumes of 2.3 M sucrose solution in PBS. 10- μ l drops of this concentrated suspension were applied on copper specimen holders of the ultracryotome, shock frozen, and stored in liquid nitrogen. Material prepared this way was used for up to 4 wk after fixation.

In some experiments platelets were embedded in gelatin after the platelets were fixed and washed three times in PBS. The pelleted platelets were suspended in a 10% gelatin (wt/vol) solution in phosphate buffer at 37°C. After 5-min equilibration at 37°C the platelets were spun down again. Supernatant gelatin was pipetted off and the remaining, highly concentrated platelet suspension was deposited as one large drop on the underside of a petri dish bottom (94/16; Creiner Labor Technik, Alphen a/d Rijn, Holland) and flattened to an \sim 0.5-mm thick film by lowering the bottom of a smaller petri dish (35/10; Creiner) onto it. The gelatin-platelet sheet was solidified in a refrigerator, and, after removing the smaller petri dish, fixed in the original platelet fixative for 30 min. The sheet was shaved off the petri dish with a razor blade and immersed in 2.3 M sucrose in phosphate buffer for 30 min. From this sheet, small trapezoid-shaped blocks were prepared, mounted onto the specimen holders, and frozen as described before. Gelatin embedding of the platelets resulted in better sectioning by the microtome and improved platelet morphology.

Cryosectioning. Cryosections of the platelet suspension were cut at -70° to -90°C according to Tokuyasu and Singer (15) with a Sorvall (DuPont Instruments, Sorvall Biomedical Div., Newton, CT) MT2B ultratome with an LTC-2 cryokit attachment. For electron microscopy 100-nm sections were layered on a formvar-carbon-coated copper grid. For light microscopy sections of \sim 200 nm were layered on an alcohol-cleaned microscope slide. The sections were cut with a dry glass knife and picked up from the edge of the knife by means of a drop of a 2.3 M sucrose solution held in a thin platinum wire loop, and thawed *en route* to grid or slide.

Immunolabeling. Immunolabeling was performed by placing small drops of the following solutions over light microscope sections on glass or under electron microscope sections on grids: (a) 2% gelatin in PBS, 10 min (to prevent background staining); (b) three times PBS plus 0.01 M glycine, 2 min (to quench free aldehyde groups); (c) for light microscopy: mixture of fluorescein-goat antifibrinogen with antiplatelet factor 4 or with anti- β -thromboglobulin, 30 min; for electron microscopy: rabbit antifibrinogen, antiplatelet factor 4, or anti- β -thromboglobulin, 30 min; (d) five times PBS, 1 min; (e) for light microscopy: rhodamine-labeled goat anti-rabbit IgG, 30 min; for electron microscopy: ferritin-labeled goat anti-rabbit IgG of PAG, 30 min; (f) several changes of PBS in 20–30 min. All antibodies were used at a concentration of \sim 25 $\mu\text{g}/\text{ml}$ in PBS. PAG was diluted with PBS before use until the red color could barely be seen. To this final dilution, in which protein A was present at a con-

centration of 0.5 $\mu\text{g}/\text{ml}$, some protein (e.g., bovine serum albumin) was added to a concentration of 1%. After immunostaining, light microscopic sections were embedded in a PBS/glycerol mixture (1:1, vol/vol) and studied with a Zeiss Photomikroskop II (Carl Zeiss, Inc., FRG) with fluorescence attachment, including a filter combination enabling us to photograph both fluorescent signals exclusively. Electron microscopic sections were stained for 10 min with 2% neutral uranyl oxalate and for 2 min with 0.2% uranyl acetate (pH 4.0) and embedded in methyl cellulose (16).

The specificity of the staining was tested with blocking experiments. Anti- β -thromboglobulin γ -globulin was serially diluted and studied for immunofluorescence of platelets. Absorption was performed with five times as much β -thromboglobulin as γ -globulin, by incubation for 1 h at 37°C and overnight at 4°C. The sample was centrifuged (2 min 10,000 *g*, 4°C) and the supernatant was used for immunofluorescence studies. Similar studies were performed for antifibrinogen with fibrinogen and fibronectin purified according to Vuento and Vaheri (36) and for antiplatelet factor 4 with purified platelet factor 4, chondroitin-6-sulfate from shark cartilage, heparin, platelet factor 4, and chondroitin sulfate (1:1) and platelet factor 4 and heparin (1:1). Chondroitin sulfate or heparin were added to platelet factor 4 to solubilize it. Absorption of anti- β -thromboglobulin with β -thromboglobulin, antiplatelet factor 4 with platelet factor 4 plus chondroitin sulfate or plus heparin and of antifibrinogen with fibrinogen abolished fluorescent staining of the platelets. Absorption of antifibrinogen with fibronectin had no effect.

RESULTS

Ultrastructure. The ultrastructure of human blood platelets has been established previously (37–39) and, in general, platelet morphology in the cryosections was very similar to that in plastic sections.

As judged from the presence of pseudopodia, the washed platelets were often activated. Dense bodies and glycogen particles were not discernible in immunocytochemically treated cryosections of platelets, even if they were fixed with up to 2% glutaraldehyde.

Immunofluorescence microscopy. Localization experiments with antifibrinogen, antiplatelet factor 4, and anti- β -thromboglobulin with the indirect method in 200-nm cryosections of washed platelets revealed a similar dotlike labeling pattern for all three antibodies with no labeling of cytoplasm or cell membrane. To prove that the antigens were localized in the same platelet, or even the same granule, we investigated their distribution with a double-immunofluorescent labeling technique. Fig. 1 shows the labeling patterns for platelet factor 4 (B) and fibrinogen (C) and for beta thromboglobulin (E) and fibrinogen (F). For all three antigens the fluorescence pattern was present over all platelets in a punctate, intracellular pattern. Labeling of cell membrane and cytoplasm was not observed. From the two sets of photographs (A-C and D-F) it is clear that the punctate fluorescence covers nearly all granulelike spots in the phase-contrast images (A-D), and that the fluorescence patterns are highly compat-

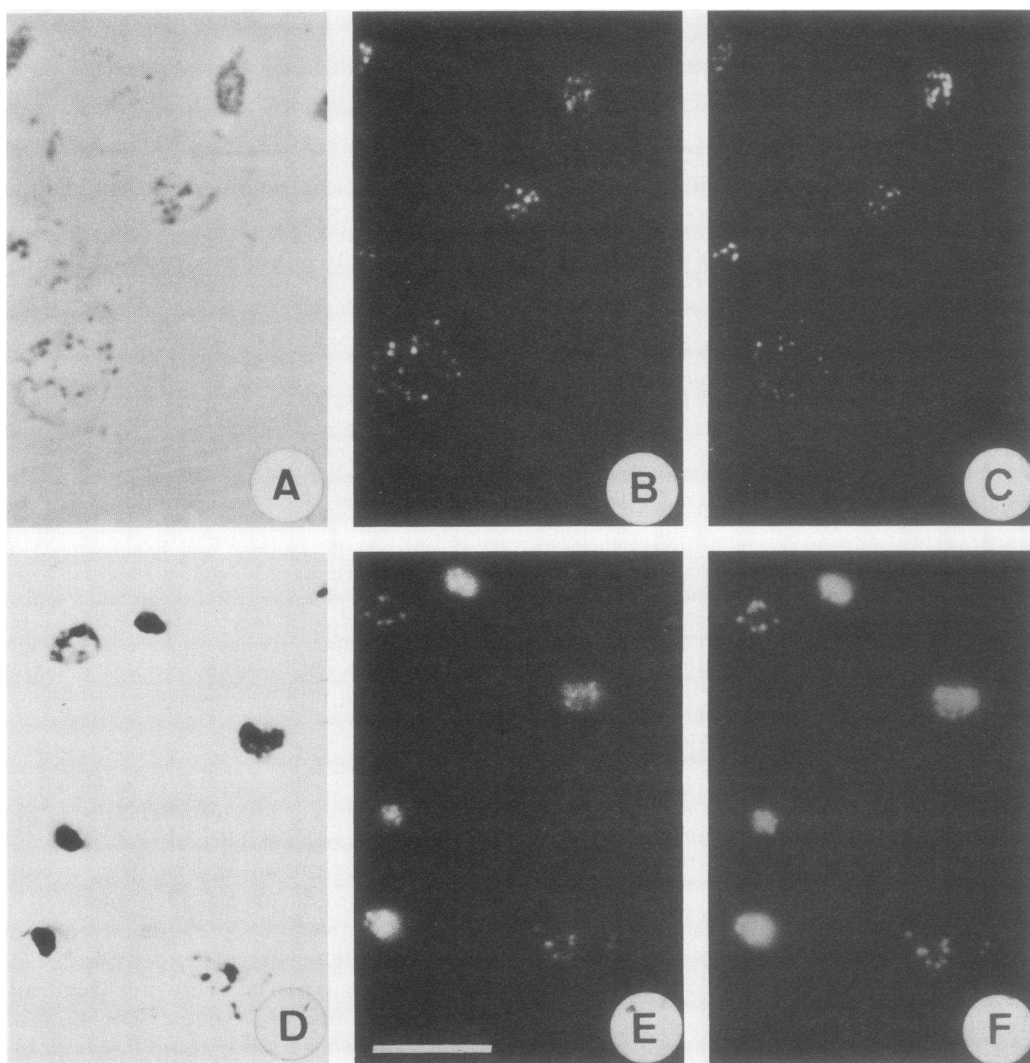


FIGURE 1 In *A* the phase-contrast image is given of a thin section of platelets double labeled with antiplatelet factor 4 visualized with rhodamine-conjugated goat anti-rabbit antibodies (*B*), and with fluorescein-conjugated goat antifibrinogen antibodies (*C*). Note the nearly complete identity of the fluorescence patterns (*upper row*). Double-label experiment to demonstrate on one platelet section the localization of beta thromboglobulin by indirect rhodamine staining (*E*) and of fibrinogen by direct fluorescein staining (*F*). Phase-contrast image of the section is given in *D*. Again a highly compatible labeling pattern is seen in *E* and *F* (*lower row*). Fixation, 2% paraformaldehyde. Magnification $\times 1.600$; bar, 10 μm .

ible, indicating that platelet factor 4, fibrinogen, and beta thromboglobulin are stored not only in the same platelet but also in the same spots. Control experiments performed with sections incubated with fluorescein-conjugated goat antifibrinogen alone or mixed with the normal rabbit IgG fraction and subsequent labeling with rhodamine-conjugated goat anti-rabbit IgG antibodies showed no rhodamine fluorescence.

Immunoelectron microscopy. In washed platelets fibrinogen was demonstrated in granules with mod-

erate electron density, the α -granules (Fig. 2 *A* and *B*), but not in the cytoplasmic matrix, mitochondria, or on the cell membrane.

Beta thromboglobulin (Figs. 3 and 4) and platelet factor 4 (Fig. 5) were localized in granules of the α -type but not in the cytoplasm, mitochondria, or on the cell membrane. These results are in agreement with the dotlike labeling patterns that we found with our immunofluorescence experiments (Fig. 1 *B*, *C*, *E*, and *F*).

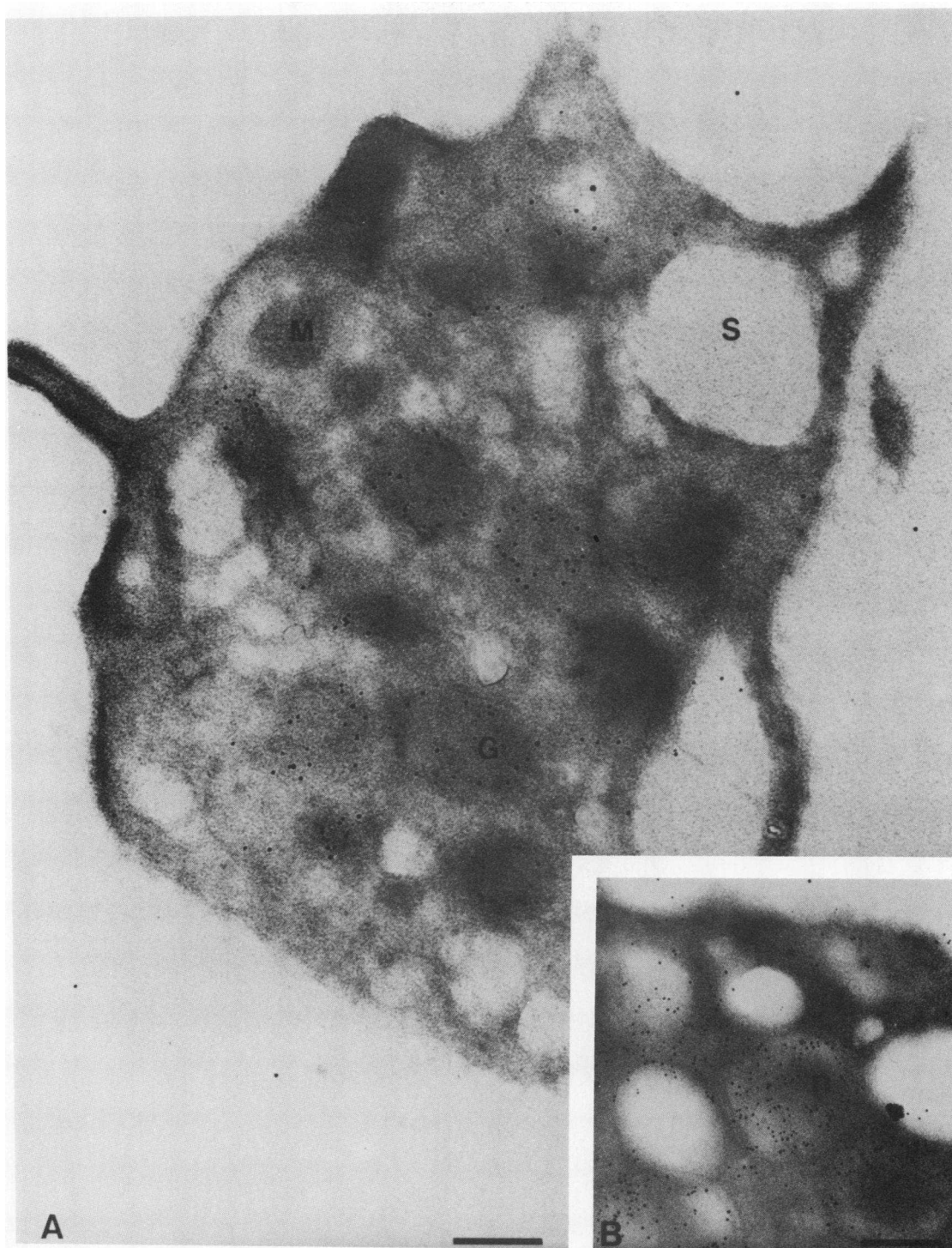


FIGURE 2 Demonstration of fibrinogen in human platelets using ultrathin cryosections incubated with antifibrinogen and labeled with PAG. Label is present over the electron lucent periphery of α -granules (G), but to a much lower extent over the dense nucleoids (N). Surface-connecting system (S), cytoplasm, and mitochondria (M) are not labeled. Magnification: A, $\times 63,000$; B, $\times 62,000$; bars, $0.2 \mu\text{m}$.

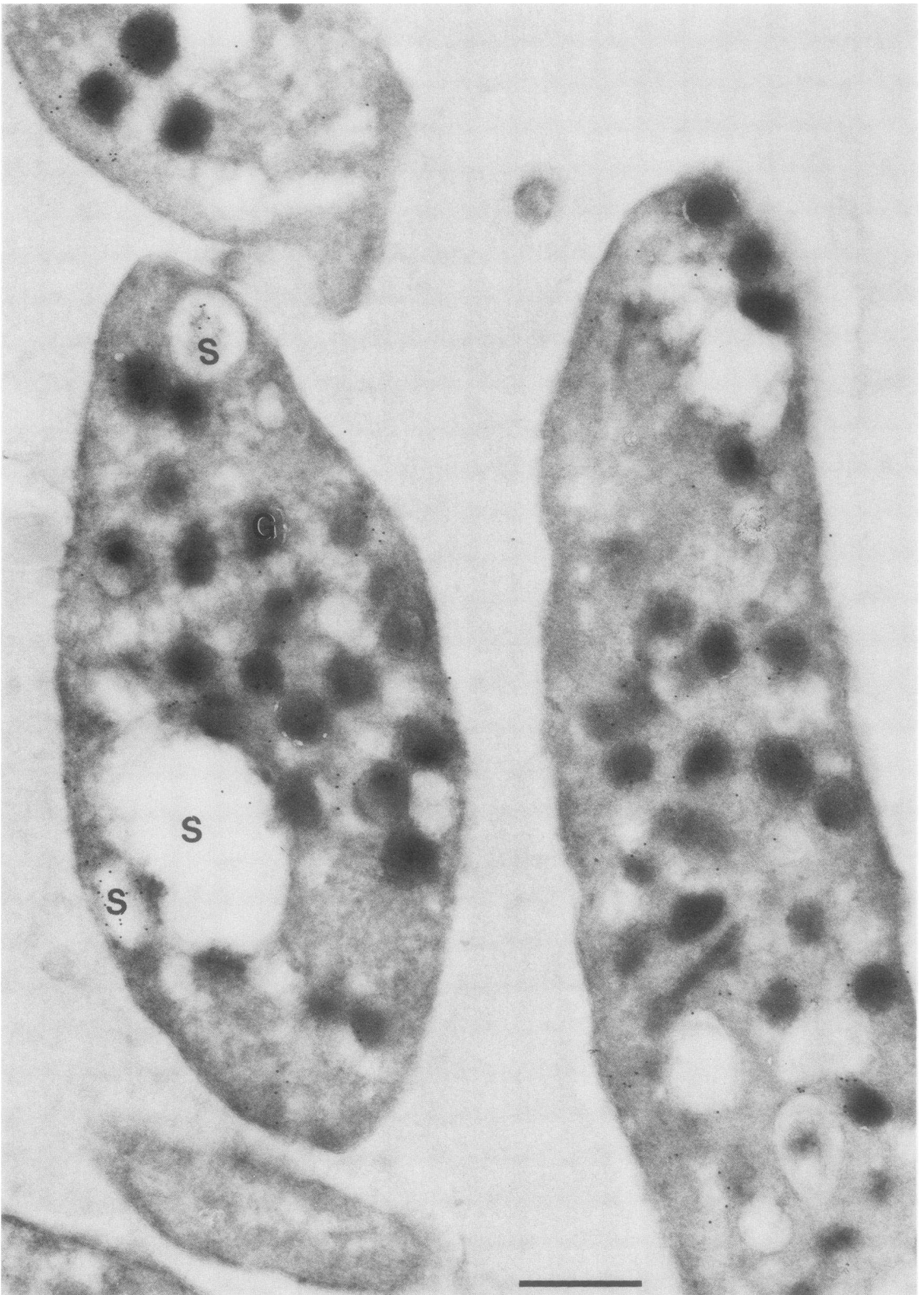


FIGURE 3 Ultrathin cryosections of freshly fixed platelets, immunostained with PAG (8 nM) for β -thromboglobulin at relatively low magnification. $\times 33,000$; bar, 0.2 μm .

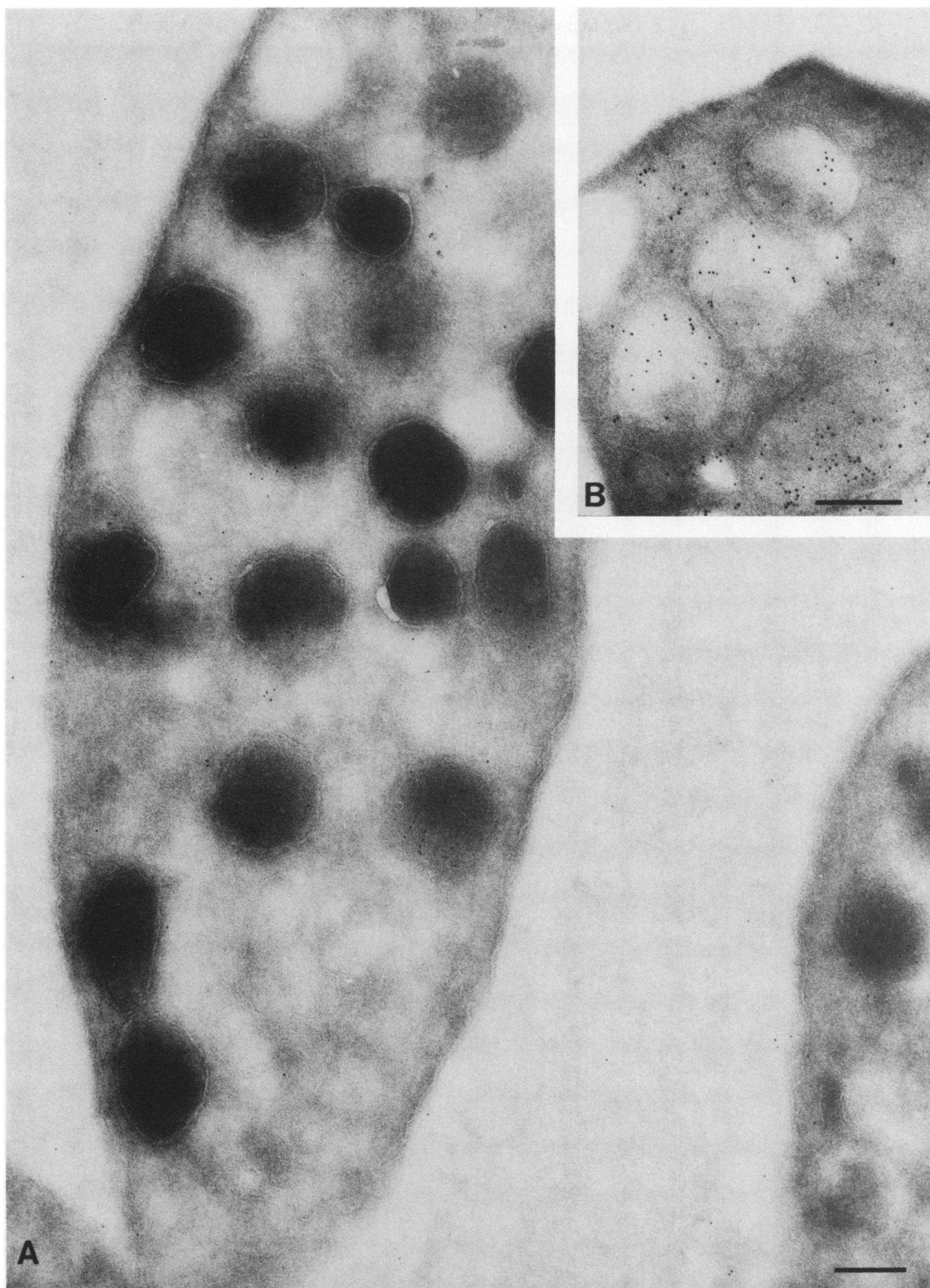
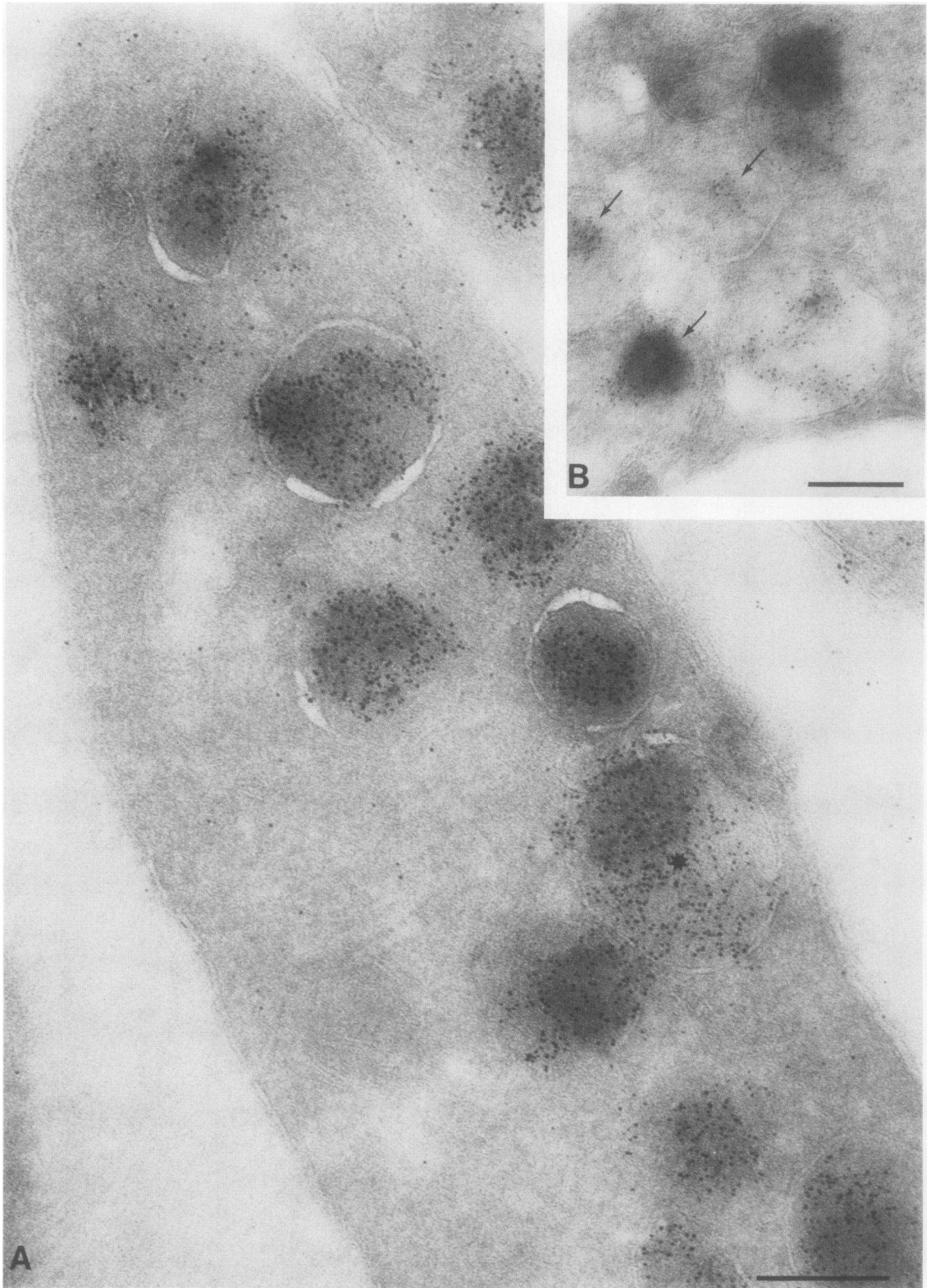


FIGURE 4 Ultrathin cryosections of freshly fixed platelets, immunostained with PAG for β -thromboglobulin. α -Granules show specific staining, but the density of label varies considerably per granule. The distribution of label within the granules seems not to be related to the nucleoids. Magnification: A, $\times 58,000$; B, $\times 70,000$; bars, $0.2\ \mu\text{m}$.



The intragranular storage pattern of the three proteins differed conspicuously. Platelet factor 4 was localized mainly in the electron-dense nucleoid (Fig. 5). Fibrinogen occupied the electron-lucent periphery of the granule content (Fig. 2 A, B), whereas for beta thromboglobulin no preferential labeling of either nucleoid or periphery of the granules were observed. (Figs. 3 and 4 A, B).

DISCUSSION

We investigated the distribution of fibrinogen, platelet factor 4, and beta thromboglobulin in lightly fixed platelets by immunolabeling of cryosections with a thickness varying from 200 nm for fluorescence microscopy, down to 100 nm for electron microscopy studies.

For immunofluorescence this procedure resulted in very good resolution, because superposition of different structures does not occur, as is the case when normal sections ($>1 \mu\text{m}$) or whole cell preparations are used. This approach enabled us in an earlier study to prove that FVIII R:Ag-containing dots were localized inside the platelets and not at the cell surface (12). In the present study we took advantage of this technique and the availability of double-labeling procedure for immunofluorescence to gain more information on the localization of fibrinogen, platelet factor 4, and beta thromboglobulin in platelets. All three proteins appeared to be distributed in an identical, intracellular, punctate pattern, which was similar to that described for FVIII R:Ag (12).

Another advantage of the fact that the section thickness is below the diameter of the cells as well as most of its membrane-bound compartments, is that it gives optimal accessibility of intracellular immunoreactive sites, so that treatment to disrupt the membranes are no longer needed. Such treatments are harmful to the ultrastructure and may cause artifacts, such as a redistribution of antigens. This might have happened in other studies, where fibrinogen, in contrast to the present results, appeared in the cytoplasm but not in the α -granules (14). Since we worked with properly fixed cells, the reactive dots observed in fluorescence experiments could easily be recognized as α -granules with the electron microscopic immunotechnique.

It is evident that all three substances, fibrinogen, platelet factor 4, and beta thromboglobulin, are lo-

calized in α -granules, and that virtually each granule contains all three proteins. Within the granule the antifibrinogen reaction occurred mainly in the periphery. Apparently this distribution is not caused by impaired antibody penetration into the electron-dense granule core (nucleoid), since antiplatelet factor 4 did react with the nucleoid, whereas in that case the granule periphery stained less. Therefore, we concluded that in the sections fibrinogen is predominantly localized in the electron-lucent periphery of the α -granules and that platelet factor 4 occurs mainly in the central part. Beta thromboglobulin exhibited an equal distribution over both parts.

The antibody raised against platelet factor 4 was made against a platelet factor 4-chondroitin sulfate complex. To exclude the possibility that contaminating antibodies against chondroitin sulfate might be present, absorption of the antiplatelet factor 4 was performed with a platelet factor 4-heparin complex as well as with a platelet factor 4-chondroitin sulfate complex. In both instances immunofluorescent staining was fully abolished, indicating that no antibodies against the proteoglycan carrier occurred in the affinity-purified antiplatelet factor 4 (40).

Data from the literature indicate that the granule nucleoid contains a large amount of proteoglycan (41, 42). The differential localization in the granule may therefore be explained by assuming that the larger fibrinogen molecule (mol wt 340,000) is excluded from the dense proteoglycan accumulation, whereas the much smaller beta thromboglobulin molecule (mol wt 36,000) may penetrate into this area.

Theoretically, this apparent heterogeneity of proteins, within an α -granule could be the result of preferential washout of the proteins. However, the complementary location of fibrinogen and platelet factor 4 in the presence of a homogeneously distributed third factor, β -thromboglobulin, make the explanation of the phenomenon less likely.

We never noticed dense bodies in thin frozen sections, even if we raised the glutaraldehyde concentration to 2%. Their presence in similarly fixed and subsequently resin-embedded platelets (5) proved that they are lost as recognizable entities after cutting. Probably this is due to leakage of dense body constituents during immunocytochemical treatment, which makes them indistinguishable from α -granules, and yields the unlabeled granules that we occasionally find.

FIGURE 5 Ultrathin cryosections of freshly fixed platelets, immunostained with PAG for platelet factor 4. Immunoreactivity occurs in α -granules. Sometimes label is evenly distributed over the entire granules (asterisk) but often the dark nucleoids are stained preferentially (arrows). Other parts of the cells are not labeled significantly. Magnification: A, $\times 106,000$; B, $\times 77,000$; bars, $0.2 \mu\text{m}$.

It is clear that the dense body contents cannot be immobilized by aldehydes, but need a mechanical fixing medium such as epoxy-resins.

In experiments where platelets were stimulated to secrete, platelet factor 4 and beta thromboglobulin were released in parallel (9, 43, 44), which points to a common storage site. However, Zucker et al. (9) found different release patterns for fibrinogen and beta thromboglobulin, suggesting a possibly different storage site for beta thromboglobulin and platelet factor 4 compared with fibrinogen. Also FVIII R:Ag, a protein also reported to be localized in α -granules (12, 17), showed release kinetics similar to fibrinogen, but different from beta thromboglobulin. The common localization of fibrinogen, as reported here, has implications for the understanding of the platelet secretion reaction. Release differences between these proteins (9, 44) might therefore result from differential storing of secretory proteins in the open canalicular system.

The occurrence of fibrinogen, platelet factor 4, and beta thromboglobulin in the same granules is in agreement with biochemical experiments, in which cell fractionation was used as a technique for the localization of proteins in platelets (1, 4, 8, 10, 13). Fibrinogen (10, 13), platelet factor 4 (8), and beta thromboglobulin (7) were found in subcellular fractions enriched for α -granules and not in the membrane fraction or soluble fraction. Our results complement the known biochemical data in that they show that all platelets and virtually all α -granules contain the same substances and that it is unlikely that α -granule subpopulations exist. This, in turn means that the reported nonparallel release cannot be attributed to graded secretion steps and necessarily must be a result of interactions that take place after the secretion process has induced the granules to be exocytosed.

It is unknown how the secreted α -granule substances behave between leaving the secretion granule and before appearing in the platelet-suspending medium. Ginsberg et al. (46) have shown that large vacuoles occurred in thrombin-treated platelets, which contained platelet factor 4 before it was secreted from the cell. Specific retention of some proteins in these vacuoles might explain nonparallel release.

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