THE CONTENT AND FATE OF PLATELET ALPHA GRANULES

by

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TABLE OF CONTENTS

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ABSTRACT

We investigated the distribution of β-thromboglobulin (βTG), platelet Factor 4 (PF4), and fibrinogen in unstimulated human platelets by several immunocytochemical techniques. All three substances were localized in the majority of platelet alpha granules either by immunoperoxidase methods on saponin-treated platelets or by colloidal gold immunoconjugates on frozen thin sections. Double-label experiments on frozen thin sections showed the presence of pairs of these three proteins in the same granules, although not every granule contained both substances.

As part of the above studies, we also determined the optimal conditions for preparing and fixing platelets for immunocytochemistry. We systematically compared platelets obtained from blood dripped directly into fixative or anticoagulated blood. We determined that temperature was the most important variable. That is, if the blood was mixed with an anticoagulant and maintained at 37°C for 1 h before fixation, there was the same number of unactivated platelets as in samples from blood immediately fixed. The same intracellular localization of βTG, PF4, and fibrinogen was found in platelets prepared by the two techniques. The results of the plasma membrane localization of antigen, however, did differ with platelets prepared by these two methods and were also influenced by the presence of the permeabilizing agent, saponin. Exposure to saponin altered both the specific and nonspecific labeling of the plasma membranes.

Thus, we have clearly demonstrated at the ultrastructural level using two immunocytochemical procedures on platelets prepared by several methods, the presence of βTG, PF4, and fibrinogen in platelet alpha granules.

The redistribution of β-thromboglobulin (βTG), platelet Factor 4 (PF4), and fibrinogen from the alpha granules of the platelet after stimulation with thrombin was studied by morphologic and immunocytochemical techniques. The use of tannic acid stain and quick-freeze techniques revealed several thrombin-induced morphologic changes. First, the normally discoid platelet became rounder in form, with filopodia, and the granules clustered in its center. The granules then fused with one another and with elements of the surface-connected canalicular system (SCCS) to form large vacuoles in the center of the cell and near the periphery. Neither these vacuoles nor the alpha granules appeared to fuse with the plasma membrane, but the vacuoles were connected to the extracellular space by wide necks, presumably formed by enlargement of the narrow necks connecting the SCCS to the surface of the unstimulated cell. The presence of fibrinogen, βTG, and PF4 in corresponding large intracellular vacuoles and along the platelet plasma membrane after thrombin stimulation was demonstrated by immunocytochemical techniques in saponin-permeabilized and non-permeabilized platelets. Immunocytochemical labeling of the three proteins singly and in pairs on frozen thin sections of thrombin-stimulated platelets confirmed these findings and showed that all three proteins reached the plasma membrane by the same pathway. We conclude that thrombin stimulation of platelets causes at least some of the fibrinogen, βTG, and PF4 stored in their alpha granules to be redistributed to their plasma membranes by way of surface-connected vacuoles formed by fusion of the alpha granules with elements of the SCCS.

DISSERTATION INTRODUCTION

Platelets contain a variety of organelles, the most numerous of which are storage granules containing substances that may be released during platelet secretion. These granules may be subdivided into four populations--alpha granules, dense granules, lysosomes, and peroxisomes--based upon their different densities, their ultrastructural appearances, and their specific contents.

Prior to 1955, it was not known that platelets release stored substances. The first evidence for this was obtained by Zucker and Borelli (1) and Born and Gillson (2) who noted that platelets absorb serotonin from plasma and release this substance during clotting of platelet-rich plasma or after thrombin stimulation of platelets. Grette (3) carried out a detailed study in 1962 of serotonin release from pig platelets after thrombin stimulation and demonstrated by fluorometric procedures that exposure of platelets to thrombin led to serotonin release. In addition, he observed that amino acids, fibrinogen, and adenine nucleotides were secreted. Grette postulated that the effect of thrombin on platelet plasma membranes was to make them permeable to Ca^{++}, and that this then intracellularly-located Ca^{++} could cause a platelet contractile event resulting in translocation of some intracellular substances to the cell exterior. By transmission electron microscopy, two separate investigations (4,5) demonstrated that following thrombin interaction with platelets, few breaks were seen in the platelet plasma membranes. This indicated that release of cellular material was more specific than had earlier been apparent and was not accomplished by membrane rupture.

Buckingham and Maynert (6) showed in 1964 that platelets released serotonin from their granules after incubation with thrombin. Further support for the selectivity of the platelet "release reaction" (a term coined earlier by Grette) was given by Holmsen (7) and Ireland (8) who showed that thrombin-stimulated platelets released metabolically-inactive nucleotides and retained metabolically-active nucleotides, indicating that there were two pools of these platelet substances. At approximately the same time, acid hydrolase activity was also found in platelets: Zucker and Borelli (9) noted that all of the acid β-glycerophosphatase activity and some of the p-nitro-phenylphosphatase activity (10) present in human serum were derived from platelets.

The technique of subcellular fractionation became widely used during the late 1960s and early 1970s as a method of localizing substances to various platelet fractions. This procedure is hampered by two principal disadvantages: 1) organelles may be disrupted during the fractionation process and their contents redistributed, and 2) contaminant organelles may be present in various fractions. Nevertheless, significant data regarding the distribution of platelet constituents were obtained using this technique. Holmsen and Day (11) studied the release and retention of various platelet components after thrombin stimulation and demonstrated that markers for mitochondria, cytoplasm, and membranes were retained while markers for granules were released into the extracellular medium. An extension of this study indicated that the nonmetabolic, releasable pool of adenine nucleotides was present with serotonin in dense granules while the metabolic pool was not (12). Marcus et al (13) isolated human platelet granules and demonstrated that they contain several lysosomal enzymes--acid phosphatase,

β-glucuronidase, and cathepsin--as well as a soluble catalase whose origin could not be determined. Using a discontinuous sucrose gradient, Siegel and Lüscher (14) showed that a fraction containing most of the acid phosphatase and platelet factor 3 activity could be partially separated from a fraction containing the majority of β-glucuronidase and cathepsin activity . They concluded that β-glucuronidase and acid phosphatase may be contained within different granules, but Holmsen and Day (11) suggested that some enzymes within the same granules might be more tightly bound to the membrane than others. Walter et al (15) and Kaulen and Gross (16) demonstrated several acid phosphatases in human platelet homogenates, and concluded that platelet β-glycerophosphatase is lysosomal. Two morphologically distinct types of platelet granules were reported in pig and human platelets by Morgenstern et al (17) both of which contained acid phosphatase activity but differed in their content of other lysosomal enzymes. Fibrinogen was found in platelet lysates (18) and was believed by Nachman et al (19) to be a platelet granule constituent. Concurrent with these early subcellular fractionation experiments, ultrastructural analysis of platelets localized serotonin in granules which had a "bull's eye" appearance (20-24).

Until early 1970s, alpha granules had generally been considered to be lysosomal in nature, although Siegel and Lüscher (14) were the first to suggest that alpha granules are not "typical lysosomes". More advanced subcellular fractionation techniques developed by Broekman et al (25) on human platelets clearly differentiated acid hydrolase-associated structures from a relatively pure alpha granule fraction. These investigators noted acid hydrolase activity in membranous vesicles and anisometric bodies besides granules, and concluded that there was morphological

and biochemical heterogeneity among platelet lysosomes. Concomitantly, similar results were obtained by Bentfeld and Bainton (26) using cytochemical procedures to identify two lysosomal constituents--aryl sulfatase and acid phosphatase--at the ultrastructural level in rat platelets, and later, in human platelets (27). Reaction product for these substances was contained in granules morphologically different from alpha granules. These investigations, utilizing biochemical and morphological techniques, established the nonidentity of lysosomes and alpha granules. Another investigation by Da Prada et al (28) using subcellular fractionation procedures showed that platelet Factor 4 was contained in different fractions than 5-hydroxytryptamine (serotonin). Subsequently, additional subcellular fractionation experiments have localized fibrinogen, platelet Factor 4, low-affinity platelet Factor 4, β-thromboglobulin, platelet-derived growth factor, fibronectin, and Factor VIII-related antigen to the platelet alpha granule fraction (29-35). The study by Ryo et al (33), for example, showed the distribution of platelet Factor 4 in an alpha granule-enriched fraction as opposed to a dense granule fraction containing ^{14}C-serotonin or the granule fraction containing β-glucuronidase. Knowledge of a fourth platelet granule population was provided by Breton-Gorius and Guichard (36), who utilized enzyme histochemistry with alkaline diaminobenzidine medium to reveal small catalase-reactive granules with a distribution similar to that of dense bodies--the microperoxisomes. By the mid-1970s, then, the results of these investigations strongly suggested the existence in the platelet of four types of storage granules with different contents.

By the late 1970s, two other methodological approaches were used to further dissect platelet granule contents. One approach involved the

study of patients with various platelet granule defects. Weiss et al (37) studied a group of patients with storage pool disease, characterized by a decrease in number and contents of platelet dense granules. In eleven patients, these investigators found only a deficiency of dense granules and dense granule substances and in another group of seven patients, they found variable deficiencies of alpha granules and their substances as well as dense granule defects. All of the platelet samples were normal with respect to acid hydrolase levels. Gerrard et al (38) investigated two patients with the gray platelet syndrome, and observed deficient numbers of platelet alpha granules by morphologic techniques. These investigators and Nurden et al (39), also observed by biochemical means a deficiency of various substances previously localized to platelets or platelet subcellular fractions of alpha granules. This approach strengthened the conclusions drawn from subcellular fractionation data; that is, lysosomal granules and other platelet granules stored different substances. The second approach utilized immunofluorescence as a tool to define whether or not substances were localized to platelets; this technique, however, does not permit the differentiation of various granule populations due to its insufficient resolution. Nevertheless, by the late 1970s and early 1980s, many antigens had been localized to platelets using this technique: Factor V (40), Factor VIII-related antigen (41, 42), fibronectin (42, 43) platelet Factor 4 (42, 44, 45), and β-thromboglobulin (45). Ginsberg et al (44) studied the redistribution of platelet Factor 4 after thrombin stimulation of platelets. They noted that the pre-stimulus pattern of immunofluorescence which was punctuate in distribution changed to large masses after thrombin stimulation, many of which disappeared by the time secretion was virtually complete.

From these investigations, performed over a three decade period, the concept gradually evolved of the platelet as a cell containing a variety of storage granules, each with its unique constituency of proteins and enzymes, some of which may be secreted after appropriate stimulation. Yet to be definitively shown, however, was the presence of particular substances within morphologically defined granules at the electron-microscopic level. Elucidation of this question required the development and characterization of specific immunocytochemical probes for putative alpha granule substances (detailed in Chapter 1 of this dissertation). The demonstration of the presence of three secretable platelet proteins--β-thromboglobulin, platelet Factor 4, and fibrinogen--in platelet alpha granules (documented in Chapter 2 of this work) and their redistribution to the extracellular space and the platelet plasma membrane after thrombin stimulation (documented in Chapter 3 of this work) further extend our knowledge of this complex cell. The fourth and final chapter of this dissertation documents the preliminary immunocytochemical analysis of β-thromboglobulin and platelet Factor 4--two platelet-specific proteins--in the human megakaryocyte. In order to unequivocally prove that platelet substances are synthesized in the megakaryocyte, it is necessary to demonstrate their presence in the protein synthetic apparatus of this cell (i.e. rough endoplasmic reticulum and the Golgi complex). It is entirely possible, for example, that fibrinogen, known to be synthesized by the liver, is endocytozed and stored in megakaryocytes and not actually synthesized there. We have been able to show that these proteins are contained in at least some megakaryocyte alpha granules. Subsequent work will determine the time of synthesis of these proteins, their site(s) of packaging, their distribution, and their fate in this unique bone marrow cell.

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Chapter 1

PREPARATION OF β-THROMBOGLOBULIN AND ANTI-β-THROMBOGLOBULIN

MATERIALS AND METHODS

Preparation of β-thromboglobulin (βTG). βTG was purified from freshly obtained platelet pheresis products (approximately 1 x 10^{12} platelets) according to the method of Moore and Pepper (1), except that a Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, N.J.) column was used in place of the Sepharose 4B column. Fractions from the peak found to contain βTG by immunodiffusion analysis were pooled and applied to a Sephadex G-200 (Pharmacia) column. Fractions from each peak obtained from the G-200 column were pooled and tested for βTG by immunodiffusion analysis, and the pool containing βTG was then dialyzed overnight at $4^{o}C$ in 25 mM Tris-HCL buffer at pH 8.8. A second preparation of βTG was kindly supplied by Dr. D.S. Pepper of the Blood Transfusion Center, Royal Infirmary, Edinburgh, Scotland.

Preparation of antisera to βTG. Two New Zealand white rabbits were each immunized intracutaneously in 7-10 sites in the flank with 0.5 mg of βTG in 1.0 ml of Freund's complete adjuvant (Gibco Co., Grand Island, N.Y.). One month later, the rabbits were given intracutaneous injections of 0.25 mg of βTG in 1.0 ml of Freund's incomplete adjuvant. Additional immunizations were repeated every 2 weeks for the next two months. The rabbits were bled several times beginning 5 weeks after the initial immunization. The sera were stored at $-20^{o}C$. Antiserum to βTG was also supplied by Dr. D.S. Pepper.

Immunodiffusion of βTG and platelet Factor 4 (PF4) against antisera to βTG and PF4. Immunodiffusion analysis was performed at $4^{o}C$ in

agarose gels (Meridian Diagnostics, Inc., Cincinnati, Ohio). Approximately 6-7 =l of serum was allowed to diffuse for 24 hr in the agarose gel against βTG, PF4, normal human plasma, normal human serum, or human platelet lysates.

RESULTS

Immunodiffusion of βTG and PF4 against antisera to βTG and PF4. Precipitin lines were evident between βTG antigen (Fig. 1,G) and anti-βTG antiserum either supplied by Dr. D.S. Pepper (Fig. 1,A) or prepared in our laboratory (Figs. 1,B and 1,E). A wide line of identity, sometimes separable into two lines, was evident between the two antisera (Figs. 1,A and 1,B). This pattern was previously noted by Moore and Pepper (1) who suggested that this was due to polymorphism. Neither anti-PF4 antiserum (Figs. 1,C and 1,F) nor normal rabbit serum (Fig. 1,D) yielded an immunoprecipitin line with βTG. Normal human plasma also failed to yield an immunoprecipitin line with anti-βTG antiserum (not shown). Platelet lysates yielded a double immunoprecipitin line with anti-βTG antiserum (not shown).

Double precipitin lines between βTG prepared in our laboratory (Fig. 2,A and 2,C) or βTG supplied by Dr. D.S. Pepper (Fig. 2,D and 2,B) and anti-βTG antiserum supplied by Dr. D.S. Pepper (Fig. 2,E) were clearly evident. The double line immunoprecipitin pattern may be due to the hydrophobicity of the purified antigen, which might diffuse as either monomers or aggregates in isotonic agarose buffers, and is therefore probably not due to the presence of a second, unrelated protein. Further evidence of the purity of the βTG is the absence of an immunoprecipitin line between plasma and anti-βTG, and the absence of PF4, fibrinogen, or other α-granule proteins in our preparation.

A precipitin line was evident between PF4 antigen (Fig. 3,G) and anti-PF4 antiserum (Figs. 3,A and 3,E), but not between PF4 antigen and normal rabbit serum (Fig. 3,D), anti-lysozyme antiserum (Fig. 3,F)

or anti-βTG antiserum either supplied by Dr. D.S. Pepper (Fig. 3,B) or prepared in our laboratory (Fig. 3,C). Normal human serum and extracts of human platelet lysates yielded single immunoprecipitin lines with anti-PF4 antiserum (not shown).

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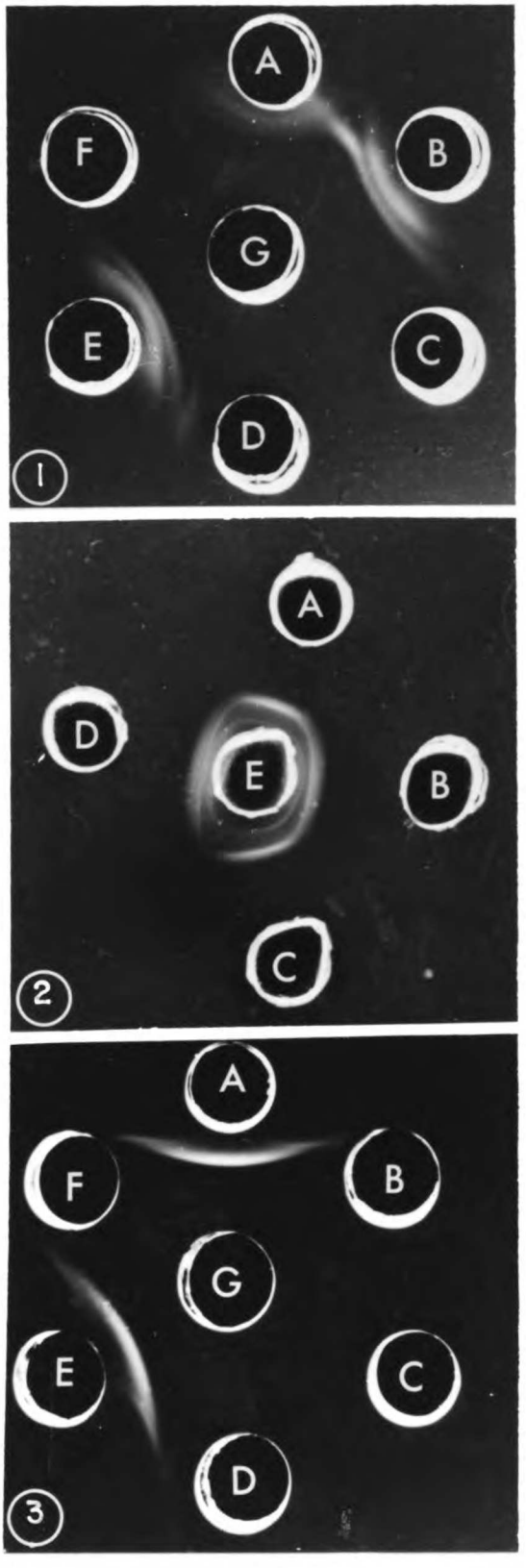
FIGURE LEGENDS

Figure 1. Immunodiffusion of platelet βTG against antisera to βTG and PF4. (A) anti-βTG antiserum (supplied by Dr. D.S. Pepper); (B) anti-βTG antiserum (prepared in our laboratory); (C) anti-PF4 antiserum; (D) normal rabbit serum; (E) anti-βTG antiserum (prepared in our laboratory); (F) anti-PF4 antiserum; and (G) βTG, (prepared in our laboratory).

Figure 2. Immunodiffusion of antisera to βTG against platelet βTG. (A) βTG, (prepared in our laboratory); (B) βTG (supplied by Dr. D.S. Pepper); (C) βTG (prepared in our laboratory); (D) βTG (supplied by Dr. D.S. Pepper); (E) anti-βTG antiserum (supplied by Dr. D.S. Pepper).

Figure 3. Immunodiffusion of platelet PF4 against antisera to βTG and PF4. (A) anti-PF4 antiserum; (B) anti-βTG antiserum (supplied by Dr. D.S. Pepper): (C) anti-βTG antiserum (prepared in our laboratory); (D) normal rabbit serum; (E) anti-PF4 antiserum; (F) antiserum to lysozyme; and (G) PF4.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Chapter 2

IMMUNOCYTOCHEMICAL DEMONSTRATION OF β-THROMBOGLOBULIN, PLATELET FACTOR 4, AND FIBRINOGEN IN ALPHA GRANULES OF UNSTIMULATED PLATELETS

INTRODUCTION

Platelets are small anucleate blood cells derived from the fragmented cytoplasm of polyploid megakaryocytes of bone marrow. The most abundant organelles in the platelet are granules synthesized during the maturation of the megakaryocyte. The granules appear to comprise several populations (1): alpha granules, dense bodies, lysosomes, and peroxisomes. Using subcellular fractionation of platelets, alpha granules have been shown to contain platelet-specific proteins, cationic proteins, coagulation factors, and other glycoproteins (see reviews, refs. 2, 3). Although these substances have been localized in the alpha granule fractions by this technique (4-13), these data must be interpreted with caution because of two well recognized disadvantages: the possibility that fractions contain contaminants and the potential for disruption of organelle membranes and consequent redistribution of proteins. Factor VIII-related antigen (FVIIIR:Ag), platelet Factor 4 (PF4), β-thromboglobulin (βTG), fibronectin, and Factor V have been localized by means of immunofluorescence techniques to platelets (14-20). Studies of platelets from patients with granule abnormalities such as the gray platelet syndrome, characterized by a relative deficiency of alpha granules, and storage pool disease, a disorder typified by a decrease in both number and contents of dense granules, have contributed to the elucidation of platelet alpha granule contents (21-23).

The biological functions of many of the platelet proteins, once they are secreted to plasma, remain unclear. βTG and PF4 have not been detected in a blood cell other than the platelet and are probably limited to the megakaryocyte-platelet lineage. Human βTG is known to bind to

bovine aortic endothelial cells, to inhibit prostacyclin (PGI_2) production (24), and to have chemotactic activity for fibroblasts (25). PF4 has been shown to have anti-heparin and chemotactic activity in vitro (25, 26-28), and inhibitory activity against collagenase (29). Synthesis of PF4 was recently demonstrated biochemically in a rabbit bone marrow culture system enriched with megakaryocytes (30). Platelet-derived growth factor is known to stimulate the proliferation of arterial smooth muscle cells and fibroblasts in tissue culture (31) and to be chemotactic for fibroblasts (25). The functions of released platelet FVIIIR:Ag and albumin are unknown. Fibrinogen is an essential cofactor for aggregation and is believed to bind to a specific membrane receptor in response to platelet activation (32). Fibronectin and thrombospondin also bind to the surface of activated platelets but the function of these proteins is unknown (33, 34).

The ultrastructural localization of proteins in platelet alpha granules has awaited the development of appropriate immunocytochemical techniques. Using several new techniques -- immunoperoxidase methods on saponin-treated platelets, and labeling with colloidal gold immunoconjugates on frozen thin sections -- we have investigated the localization of βTG, PF4, and fibrinogen in normal human platelets.

MATERIALS AND METHODS

Preparation of Platelets for Morphological Study

All platelet samples were prepared from blood drawn from normal human volunteers with a 19-gauge butterfly infusion set (Abbott Hospitals, Inc., North Chicago, Il.).

IMMEDIATELY FIXED PLATELETS: All beakers and other glassware were treated with Desicote^{R} (Beckman, Fullerton, Ca.) before use to prevent adherence of cells. Ten ml of blood was dripped from the needle into a beaker containing 90 ml of fixative comprised of 1.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) in 0.1 M Na-cacodylate buffer (pH 7.4) containing 1% sucrose. To determine the effect of the temperature of the fixative on platelet shape, aliquots of blood were dripped into fixative maintained at 4^{o}C, 22^{o}C, or 37^{o}C. After 30 min or 1 h, the mixture was spun at 150 g for 20 min in a Sorvall centrifuge with swinging buckets at room temperature. Platelet counts by hemocytometer showed that 60-90% of the platelets were recovered in the supernatant. The supernatant was spun at 1,700 g for 10 min, and the platelet pellet washed three times in 0.15 M phosphate buffer containing 3% sucrose (pH 7.4), in preparation for either transmission electron microscopy (TEM) or scanning electron microscopy (SEM). Platelets prepared in this way will be referred to as immediately fixed platelets.

FIXATION OF PLATELETS FROM ANTICOAGULATED BLOOD: The above procedure has a major disadvantage in that immediately fixed platelets cannot be used for physiological studies, such as those on platelet activation. These studies are generally performed on platelets from blood drawn into anticoagulant such as acid-citrate-dextrose (ACD) or sodium citrate which are then washed and concentrated at room temperature. In order to determine the effect of temperature on platelets from anticoagulated blood, we stored mixtures of blood and anticoagulant at various temperatures for 1 h before fixation. Blood was drawn into syringes and added to tubes containing 1/10 vol of 3.8% sodium citrate or 1/6 vol of ACD (0.085 M trisodium citrate, 0.065 M citric acid, 2.0% dextrose, pH 4.5).

After 1 h at 37°C, 22°C, or 4°C the blood was fixed for 1 h in glutaraldehyde fixative maintained at the same respective temperatures. The platelets were then washed as described above for immediately fixed platelets.

FIXATION OF WASHED PLATELETS: In the above section, anticoagulated blood was added directly into fixative without washing. Experimentally, it is frequently necessary to wash platelets from anticoagulated blood in order to perform further studies. The platelet preparatory method described below is the recommended procedure. All procedures through fixation were done at 37°C. All solutions contained prostaglandin (PGE_1) (Sigma, St. Louis, Mo.) in a final concentration of 5-10 μg/ml to inhibit platelet activation. Immediately after being drawn, blood was placed in 50-ml polycarbonate tubes with ACD and centrifuged for 10 min at 120 g. The platelet-rich-plasma (PRP) was transferred to clean tubes and centrifuged for 10 min at 2,300 g. The resultant pellets were washed twice in 0.14 M NaCl, 0.02 M Hepes, 0.006 M dextrose, 0.001 M EDTA (pH 6.6) using the same centrifugation conditions. The platelet pellets were resuspended in 0.01 M Tris-HCl, 0.14 M NaCl, 0.006 M dextrose, 0.001 M EDTA (pH 7.4). Either immediately or after being stored for 30 min at 37°C, the pellets were fixed in glutaraldehyde fixative.

Preparation of Platelets for Electron Microscopy

TRANSMISSION ELECTRON MICROSCOPY: Platelets prepared by the three methods described above were postfixed in 1% OsO_4 for 1 h. Samples were then exposed to uranyl acetate for 1 h, dehydrated in a graded series of ethanol, infiltrated with propylene oxide:Epon first in a ratio of 1:1 and then in a ratio of 1:2, and finally infiltrated with 100% Epon. Thin sections were cut, stained with either uranyl acetate and lead

citrate or lead citrate alone, and examined with a Siemens 101 transmission electron microscope.

SCANNING ELECTRON MICROSCOPY: Both immediately fixed platelets and platelets fixed after anticoagulation of the blood were washed three times in 0.15 M phosphate buffer (pH 7.6) and placed on coverslips coated with poly-L-lysine (Sigma). The coverslips were stored in 1.5% glutaraldehyde overnight at $4^{o}C$. The platelets were then dehydrated, either immediately or after postfixation in 1% OsO_4 for 1 h and exposure to uranyl acetate for 1 h. Both groups were critical-point-dried in a Polaron^{R} (Line Lexington, Pa.) critical-point-dryer immediately after dehydration, coated with approximately 200 Å of gold:palladium, and viewed with a Coates and Welter scanning electron microscope at 20 kV (35).

Immunocytochemical Procedures

Primary antisera were raised in rabbits against human proteins. Antiserum to β-thromboglobulin (βTG) was prepared in our laboratory according to published procedures (36). Antiserum to platelet Factor 4 (PF4) (26, 37) was affinity-purified and characterized by Dr. Shirley P. Levine. These two antisera were clearly distinguished from one another by immunodiffusion analysis. Antiserum to fibrinogen was affinity-purified by Dr. David R. Phillips. In control incubations, normal rabbit serum and buffer were used in place of specific primary antiserum.

Immediately-fixed platelets and washed platelets were fixed in either 2% or 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), or 8% paraformaldehyde in 0.1 M PIPES buffer (pH 7.2) for 1 hr at

37°C. Fixed platelets were washed for 5 min three times in phosphate-buffered saline (PBS) containing 50 mM NH_4Cl. The cells were soaked for 30 min in PBS containing 0.2% (Wt/Vol) gelatin and 0.005-0.1% (Wt/Vol) saponin and then incubated at room temperature for 90 min with primary antiserum diluted with the same solution. After incubation with primary antiserum, or control substance, the cells were washed three times over a period of 1 h in the PBS-gelatin-saponin solution, then incubated for 90 min with one of the following reagents, diluted in the wash solution: a) Fab fragments of sheep anti-rabbit IgG conjugated to peroxidase (Institut Pasteur, Paris, France) in a dilution of 1:50-1:100 (Fab-peroxidase); b) protein A-peroxidase conjugate (Zymed Laboratories, Burlingame, Calif.) in a dilution of 1:50-1:100 (pA-peroxidase); or c) goat anti-rabbit IgG (GAR) linked to colloidal gold (Janssen Pharmaceuticals, Beerse, Belgium) in a dilution of 1:10-1:100, depending upon which type of colloidal gold was used (GAR-G5 or GAR-G20). The platelets were washed in PBS three times over 1-h then in 0.1 M Na-cacodylate buffer (pH 7.2). They were fixed at room temperature for 30 min in glutaraldehyde fixative, washed again in Na-cacodylate buffer, and stored overnight at 4°C in the same buffer with 5% sucrose. Cells processed with colloidal gold immunoconjugates were post-fixed in 1% OsO_4, exposed to uranyl acetate, and then processed for TEM. Samples processed with immunoperoxidase conjugates were washed several times with 0.1 M Tris-HCl (pH 7.6), preincubated for 10 min with Tris buffer containing 1 mg/ml 3,3-diaminobenzidine, Grade II (Sigma) and then incubated for 10 min with diaminobenzidine containing 0.01% (vol/vol) H_2O_2. The platelets were washed well with 0.1 M Tris-HCl buffer (pH 7.6) and then with 0.1 M Na-cacodylate buffer, (pH 7.2). They were post-fixed in reduced osmium

(38). Briefly, the cells were exposed to 1% unbuffered OsO_4 for 2 min, followed by 15 min in reduced osmium, produced by adding 5 mg potassium ferrocyanide to 2 ml of 1% unbuffered OsO_4. After removal of the reduced osmium the cells were rinsed for 2 min again in 1% unbuffered OsO_4 and dehydrated, infiltrated, and embedded for TEM as described above. These samples were not exposed to uranyl acetate.

For studies of the localization of proteins to the plasma membrane, platelets were prepared as described above, except that saponin was omitted from all of the solutions used. Samples were incubated with either buffer, normal rabbit serum, anti-βTG serum, anti-PF4 serum, anti-fibrinogen serum, or rabbit anti-human IgG serum (Accurate Chemical and Scientific Corp., Westbury, N.Y.). Some samples were pre-incubated with 5% normal sheep serum before incubation with primary antiserum. Next, samples were incubated with immunoperoxidase conjugate or colloidal gold immunoconjugate.

Frozen Thin Section Procedures

Both immediately fixed and washed platelets were used in the frozen thin section studies.

Platelets fixed in 8% paraformaldehyde in 0.1 M PIPES buffer (pH 7.2), were washed well in the same buffer containing 10% (wt/vol) sucrose. Platelets fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) were washed well in the same buffer. Both groups were infiltrated for 30 min with 2.3 M sucrose, embedded in the sucrose solution, frozen, and stored in liquid nitrogen. Sections were cut on a Sorvall MT-2 ultra-microtome with a frozen thin section (FTS)/ LTC-2 attachment. The frozen thin section techniques described by

Tokuyasu (39, 40) were used, with the modifications for the use of colloidal gold described by Griffiths et al. (41). The control for single-label experiments was non-immune, purified rabbit IgG or buffer in place of the primary antiserum. Some thin sections were double-labeled with GAR-G5 and GAR-G20 as described by Geuze et al. (42). Double-label experiments were also performed with GAR-G5 and GAR-G20 in reverse order to evaluate possible technical artefacts. Buffer or non-immune, purified rabbit IgG in place of the second specific primary antiserum served as the controls.

RESULTS

Because the optimal conditions of preparing platelets for morphologic study have not been determined, we carried out the following experiments. Platelets were obtained from blood dripped directly into glutaraldehyde fixative or from blood which was anticoagulated before fixation. These preparations were then examined by TEM or SEM.

Morphological Comparison of Platelets Prepared by Different Procedures

EFFECT OF TEMPERATURE ON IMMEDIATELY FIXED PLATELETS: By TEM, immediately fixed platelets were generally disc-shaped and occasionally had a spiny projection, regardless of the temperature of the fixative. Fig. 1 a-c illustrates platelets dripped into glutaraldehyde at 37°C, 22°C, or 4°C and fixed for 1 h. Clearly, the disc shape of the platelet is retained at all three temperatures. Moreover, microtubules are visible in the platelets fixed at 4°C, as well as in those fixed at the higher temperatures (not shown). Immediate fixation seems to preserve these temperature-labile structures which are not visible in platelets

kept at low temperatures before fixation (43).

By SEM, most of the immediately fixed platelets were discoid (Fig. 1 g), and some of the discoid cells had one or more filopodia or spiny projections. A few platelets were extremely irregular in contour, and some of these also had spines. Of 100 platelets in a field covered in the scanning electron microscope, 70% were purely discoid and 30% had spines. Identical results were obtained for platelets prepared in paraformaldehyde fixative.

PLATELETS FROM ANTICOAGULATED BLOOD: By TEM, platelets prepared from blood anticoagulated with ACD were similar in shape to those prepared from blood anticoagulated with sodium citrate. Platelets from anticoagulated blood stored for 1 h at 37°C and then fixed at the same temperature were predominantly disc-shaped (Fig. 1 d) and were virtually identical in appearance to the immediately fixed samples. Many of the platelets from blood stored and fixed at 22°C were disc-shaped, but about 60% were swollen, and some of these had filopodia or spiny projections (Fig. 1 e).

The SEM appearance of the cells prepared at 22°C (Fig. 1 e) is illustrated in Fig. 1 h. There were more irregularly shaped platelets than in the immediately fixed samples. About 60% of the platelets were discoid and smooth-surfaced, whereas 40% were swollen with one or more spines. By TEM, about 95% of the platelets from blood stored and fixed at 4°C were spiny and swollen (Fig. 1 f). Thus, reducing the temperature at which the blood was stored with anticoagulant and then fixed resulted in more swollen platelets and fewer disc-shaped platelets.

Demonstration of βTG, PF4, and fibrinogen in Platelet Alpha Granules

As there is no method generally in use for identifying alpha granule

antigens in platelets at the ultrastructural level, it was necessary to adapt current techniques for accomplishing this with platelets. Antibodies against βTG, PF4, and fibrinogen were used in these experiments.

SAPONIN-TREATED PLATELETS PROCESSED WITH IMMUNOPEROXIDASE CONJUGATES: Initially, we used immediately fixed platelets for the intracellular localization of proteins. We used immunoperoxidase conjugates on cells made permeable by exposure to saponin, a sensitive technique that has been successfully used with other cells (44). Since the inclusion of even a small amount of glutaraldehyde in the fixative (for example, 2% paraformaldehyde and 0.05% glutaraldehyde) severely diminished the amount of reaction product for each antigen, we were compelled to omit glutaraldehyde and try various concentrations of paraformaldehyde.

Reaction product for βTG was present in some of the platelet alpha granules (Fig. 2 a and b) but not in others. Similar results were obtained for fibrinogen and PF4. The reaction product often filled the granules uniformly. With all three antisera, more granules per platelet were reactive: (a) when 2% paraformaldehyde was used as a fixative than when higher concentrations were used, (b) when 0.1% saponin was used as a permeabilizing agent than when lower concentrations were used; and (c) when Fab-peroxidase was used as the second marker than when pA-peroxidase was used. Plasma membranes were negative for all three antigens tested. Control preparations in which normal rabbit serum or buffer was used in place of the specific primary antiserum showed no reaction product (Fig. 2 c). Although platelets contain endogenous peroxidase (45) in the dense tubular system, we observed no reaction product for peroxidase.

FROZEN THIN SECTIONS PROCESSED WITH COLLOIDAL GOLD IMMUNOCONJUGATES: Not all alpha granules were reactive in saponin-treated platelets, possibly because the granule membranes were not sufficiently permeable to penetration of the immunoconjugates (46). To circumvent this problem and to confirm our findings by using a different marker, we performed immunocytochemical techniques on frozen thin sections of sucrose-embedded platelets. Using anti-βTG, anti-PF4, and anti-fibrinogen sera and colloidal gold immunoconjugates (GAR-G5 and GAR-G20), we demonstrated βTG (Fig. 2 d and e), PF4 (not shown), and fibrinogen (Fig. 3 a) in most, but not all, of the alpha granules. Inclusion of 0.5% glutaraldehyde in the fixative severely diminished the amount of labeling. Fixation in 8% paraformaldehyde in PIPES buffer provided optimal morphologic preservation and labeling of the alpha granules. We occasionally observed elongated granules which were usually not labeled for the three antigens tested (not shown). In control preparations, a small number of gold particles adhered nonspecifically in a scattered distribution to tissue sections.

The Effect of Saponin on the Localization of βTG, PF4, and Fibrinogen

Besides localizing fibrinogen to alpha granules by the frozen thin section technique, we were surprised to find label for this antigen on the platelet plasma membranes as well (Fig. 3 a). The gold label was probably due to the presence of plasma fibrinogen on the platelet surface at the time of fixation, since labeling was not seen with anti-PF4 serum, anti-βTG serum, or normal rabbit serum. Since we had not detected fibrinogen on the plasma membranes of saponin-permeabilized platelets using immunoperoxidase conjugates, we wondered if the saponin had decreased the labeling. Additional experiments were carried out to invest-

igate this possibility. Immediately fixed cells were processed in the absence (Fig. 3 b) and presence (Fig. 3 c) of saponin using anti-fibrinogen serum and GAR-G5 and routine TEM techniques, rather than frozen thin sections. Heavy label was seen on the plasma membranes of platelets processed without saponin. A moderate decrease in label was seen in platelets processed with saponin suggesting that saponin diminished the amount of antigen bound to the surface. Control preparations incubated with normal rabbit serum and then GAR-G5 were negative (not shown). The immunoperoxidase procedures were then repeated without saponin to see if membrane labeling with immunoperoxidase conjugates then occurred. Surprisingly, labeling occurred with normal rabbit serum (Fig. 3 d), anti-fibrinogen serum (Fig. 3 e), anti-PF4 serum (not shown), and anti-βTG serum (not shown).

In summary, in frozen thin sections processed with colloidal gold immunoconjugates, the plasma membranes were labeled after incubation with anti-fibrinogen serum. Immediately fixed platelets processed in suspension with colloidal gold immunoconjugates showed the same pattern, and the labeling was moderately decreased when the cells were exposed to saponin before incubation with anti-fibrinogen serum. In immediately fixed platelet samples processed with immunoperoxidase conjugates, regardless of the specific antiserum or control substance used, the plasma membranes were never labeled when saponin was included, and were always labeled when saponin was omitted. These findings (summarized in Table 1) show that exposure to saponin altered the labeling, both specific and nonspecific, of the platelet plasma membranes.

Studies on Washed Platelets

Because immunoperoxidase conjugates bound extensively to surface

constituents of immediately fixed, non-saponin treated platelets, such platelets were unsuitable for studies on the extracellular localization of platelet proteins (Table 2). We therefore used carefully washed platelets from anticoagulated blood to determine whether the intracellular localization of these proteins was the same as in immediately fixed platelets and to study the extracellular distribution of these substances in thrombin-stimulated platelets (see accompanying report) prepared at 37°C in the presence of an inhibitor of platelet activation. As will be documented below, this procedure is the recommended one for platelet preparation. As will be illustrated in greater detail in the accompanying paper, platelets prepared in this manner and exposed to control substances followed by either pA-pxase or GAR-G5 had minimal plasma membrane label (Table 2). All platelet preparations exposed to specific antisera or control substances followed by Fab-pxase showed considerable nonspecific plasma membrane reaction product. This binding could not be completely inhibited even when 5% normal sheep serum was used as a pre-incubation step. Exposure of washed platelets, prepared in the presence of an inhibitor of platelet activation, to rabbit anti-human IgG followed by pA-pxase resulted in positive plasma membrane reaction product; using GAR-G5 in place of pA-pxase resulted in negative plasma membrane label. This indicates that under these conditions it seems that the Fc portion of Ig on platelet plasma membranes is inaccessible to the pA-pxase probe.

SINGLE-LABEL EXPERIMENTS ON FROZEN THIN SECTIONS: We applied the immunocytochemical technique used on frozen thin sections of immediately fixed platelets to washed platelets. Platelets incubated with antiserum

to fibrinogen (Fig. 4 a and b), PF4, or βTG, and then with GAR-G5 or GAR-G20 showed negligible labeling of the plasma membranes and a granule labeling pattern similar to that in the immediately fixed platelets. As in saponin-treated, immediately fixed platelets (see previous section) or washed platelets processed with immunoperoxidase conjugates (see accompanying paper), not every platelet granule was reactive.

DOUBLE-LABEL EXPERIMENTS ON FROZEN THIN SECTIONS: To determine whether the platelet granules were homogeneous with respect to content of βTG, PF4, and fibrinogen, we double-labeled washed platelets with GAR-G5 and GAR-G20 (Fig. 4 c and d). When antisera to βTG, fibrinogen, and PF4 were used in various combinations, most of the alpha granules were labeled with both GAR-G5 and GAR-G20, although an occasional granule was labeled with only one or the other and some granules were not labeled. The results were similar when immediately fixed platelets were used instead of washed platelets (Fig. 4 e). In control preparations incubated with primary antiserum and then GAR-G5 and GAR-G20 in sequence, the granules were labeled only with GAR-G5. Thus, the second immunoconjugate caused little interference. Interference was greater when GAR-G20 was used before GAR-G5, as reported by others (42). These results indicate that βTG, PF4, and fibrinogen are present in the same granules. We have not determined, however, whether all platelet alpha granules contain all three substances.

DISCUSSION

Using immunocytochemical techniques and electron microscopy, we have shown that the proteins βTG, PF4, and fibrinogen are present in most of

the alpha granules of platelets. We demonstrated all three substances both in immediately fixed platelets and in washed platelets from anti-coagulated blood, in two ways: by using immunoperoxidase conjugates on saponin treated platelets and by using anti-IgG-colloidal gold conjugates on frozen thin sections to determine whether βTG, PF4, and fibrinogen tested in all possible combinations were present in the same granules. Most of the alpha granules contained both of the proteins tested, although occasional granules contained only one of them, and some granules contained neither one. These results imply that there may be some heterogeneity of platelet alpha granules, or that we were unable to label all of the granules due to technical limitations.

The granules in immediately fixed and washed platelets were clearly alpha granules by established morphological criteria -- that is, they were 300-500 nm in diameter and frequently had a moderately dense center and a finely granular matrix (47). We showed by two immunocytochemical methods that these granules contain βTG, PF4 and fibrinogen. However, since the relative sensitivities of our primary and secondary reagents are not known, we cannot at the present explain why all of the granules were not labeled for these proteins. Nonetheless, by double labeling for all possible pairs of proteins, we showed that they are often present in the same granules. These findings provide a conclusive ultrastructural demonstration of βTG, PF4, and fibrinogen in platelet alpha granules.

We observed a large amount of nonspecific reaction product on the plasma membranes of immediately fixed platelets processed with immunoperoxidase reagents, but without saponin. We also observed label on the plasma membranes of immediately fixed platelets processed with colloidal gold immunoconjugates and anti-fibrinogen serum, probably because plasma

fibrinogen was present on the platelet surface at the time of fixation. This nonspecific staining or labeling of the plasma membrane with immunocytochemical procedures makes immediately fixed platelets unsuitable for use in studies of localization of proteins on the platelet plasma membranes. The intracellular distribution of βTG, PF4, and fibrinogen in washed platelets processed with immunoperoxidase conjugates was similar to that in immediately fixed platelets. Moreover, the plasma membranes of washed platelets were relatively free of reaction product when treated with immunocytochemical reagents in the absence of saponin. We therefore used washed platelets exclusively in the studies on thrombin-stimulated platelets described in the accompanying report. It should be noted, however, that some of the platelets are not recovered when they are washed by centrifugation.

By comparing platelets processed with or without saponin, we found that saponin had a striking effect on labeling of the plasma membrane. When saponin was included, the labeling for fibrinogen on the plasma membranes of the platelets processed with colloidal gold immunoconjugates was slightly decreased, and there was no reaction product on the plasma membranes of platelets processed with immunoperoxidase conjugates. Thus, saponin appeared to alter surface staining, as was also suggested in a recent study on the localization of surface immunoglobulins in human B cells (48).

Studies on the localization of platelet proteins by means of subcellular fractionation have shown that the alpha granules contain fibrinogen (6,9,12), PF4 (6,7,9,11), low-affinity PF4 (49), βTG (9,12), platelet-derived growth factor (12), fibronectin (10), Factor VIII-related antigen (8), albumin (13, 23), and possibly glycoproteins II_b and III_a

(13). The amounts of many of these substances are abnormally low in platelets from patients with granule defects such as storage pool deficiency and the gray platelet syndrome (21-23). Morphological studies on the localization of platelet proteins have relied almost exclusively on immunofluorescence methods. These studies have shown that Factor V (17), PF4 (16,18,20), fibronectin (16,19), and Factor VIII-related antigen (14,16) are distributed in a punctate pattern in platelets. PF4 has been demonstrated in the cytoplasm of platelets and megakaryocytes by use of indirect immunofluorescent techniques on air-dried smears (15). PF4 and βTG have also been demonstrated in the cytoplasm of megakaryocytes and platelets by means of immunofluorescence and immunoperoxidase procedures (20). However, these techniques, because of their low level of resolution, do not reveal whether the proteins are localized in granules, whether the same granules contain more than one protein, and which types of granules contain the proteins.

Using the immunocytochemical procedures we have described, the sites of synthesis of some of these proteins, as well as their time of appearance during megakaryocyte maturation can be determined. It would also be interesting to determine whether alpha granule proteins are present in the megakaryocytes of patients with various megakaryocyte and platelet disorders. Breton-Gorius et al. (50) have suggested that a defective megakaryocyte-committed cell may be responsible for the lack of alpha granules in megakaryocytes from patients with the gray platelet syndrome. Other investigators recently described an acquired gray platelet syndrome in a patient with acute megakaryoblastic leukemia (51) and hypothesized that the local secretion of alpha granule proteins from these cells might have initiated the myelofibrosis. The observations

that guinea pig platelets and megakaryocytes contain growth factor activity (52) and that a constituent of human megakaryocytes stimulates the proliferation of bone-marrow fibroblasts (53) support this idea.

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TABLE 1

PLASMA MEMBRANE REACTION PRODUCT ON IMMEDIATELY FIXED PLATELETS USING IMMUNOCYTOCHEMICAL MARKERS ON NONPERMEABILIZED AND SAPONIN-PERMEABILIZED CELLS

Immunocytochemical Procedure	Saponin	Primary Incubation	Plasma Membrane Reaction Product
Immunoperoxidase conjugates*	yes	normal rabbit serum	-
	yes	anti-fibrinogen serum	-
	no	normal rabbit serum	+
	no	anti-fibrinogen serum	+
Colloidal gold immunoconjugates**	yes	normal rabbit serum	not done
	yes	anti-fibrinogen serum	+/-
	no	normal rabbit serum	-
	no	anti-fibrinogen serum	+

* Using either Fab fragments of sheep anti-rabbit IgG conjugated to peroxidase or protein A-peroxidase.
** Goat anti-rabbit IgG conjugated to colloidal gold (5-nm size particle) (GAR-G5).

TABLE 2

NONSPECIFIC BINDING OF IMMUNOCYTOCHEMICAL MARKERS

TO PLATELET PLASMA MEMBRANES

		Plasma Membrane Reaction Product		
Platelet Preparation	Primary Incubation	Fab-PXASE***	pA-PXASE****	GAR-G5*****
Immediately fixed Platelets	NRS*	+	+	-
	Buffer**	+	+	-
Washed platelets prepared with PGE_1	NRS	+	-	-
	Buffer	+	-	-

The above data indicate that when platelets are prepared without saponin this may result in nonspecific binding of immunocytochemical markers.

* NRS = normal rabbit serum.

** Buffer = Ca^{++}- and Mg^{++}-free phosphate buffered saline.

*** Fab-PXASE = Fab fragments of sheep anti-rabbit IgG conjugated to peroxidase.

**** pA-PXASE = protein A conjugated to peroxidase.

***** GAR-G5 = goat anti-rabbit IgG conjugated to 5-nm size colloidal gold particles.

FIGURE LEGENDS

FIGURE 1. Morphology of normal human platelets prepared by different methods. (a-c) Transmission electron micrographs of platelets prepared from blood dripped into fixative maintained at 37°C (a), 22°C (b), and 4°C (c). Note the similar proportions of discoid cells in the three samples. X 7,000. (d-f) Transmission electron micrographs of platelets drawn into ACD anticoagulant and stored at 37°C (d), 22°C (e), and 4°C (f) for 1 h before fixation. Note the preponderance of discoid cells in d as compared to the cells in e and f. X 7,000. (g, h) Scanning electron micrographs. (g) Platelets dripped into fixative maintained at 37°C. Most are discoid (d), and one has a long spine (S). X 15,000. (h) Platelets drawn into 3.8% sodium citrate anticoagulant and stored at room temperature for 1 h before fixation. These platelets are more swollen and rounded in appearance than those seen in g, and one of them has a long spine. X 15,000. By SEM, the platelets often had many surface indentations, corresponding to the openings of the surface-connected canalicular system to the external milieu. All samples were fixed for 1 h in 1.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) with 1% sucrose.

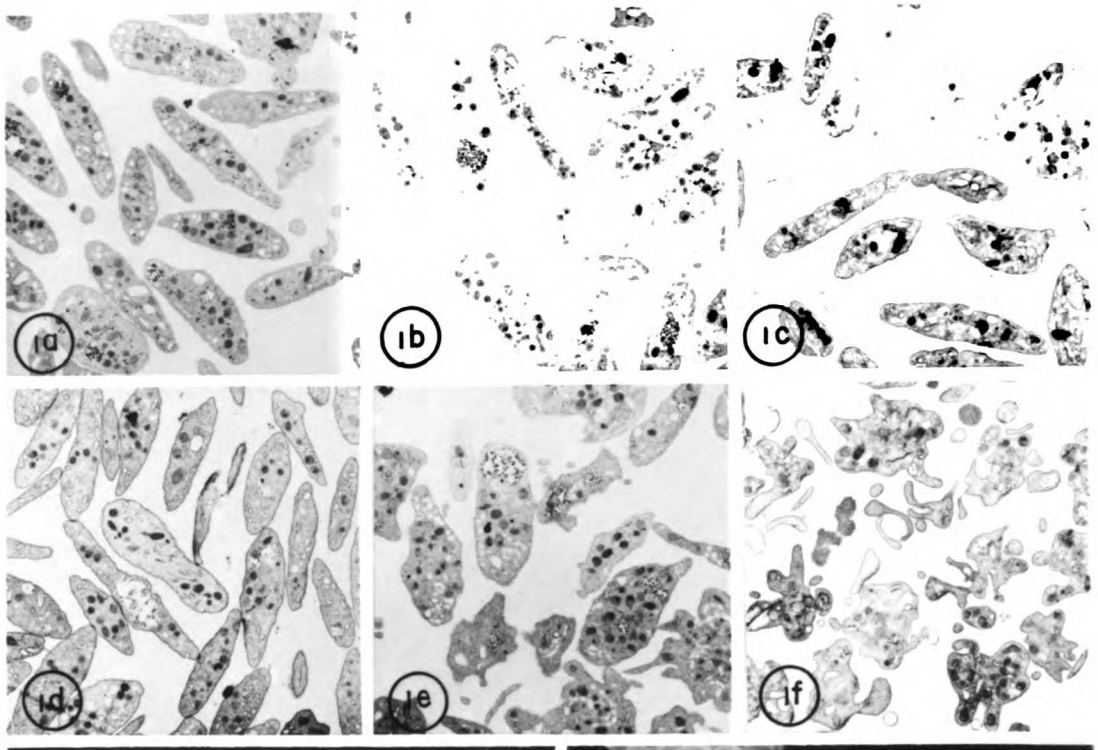
FIGURE 2. Transmission electron micrographs showing the localization of βTG in immediately fixed platelets. (a-c) Platelets fixed in 4% paraformaldehyde, treated with 0.1% saponin, and processed with immunoperoxidase conjugates. (a) Platelets incubated with anti-βTG serum and then Fab-peroxidase. Reaction product is present in some of the alpha granules but not in others (arrows) and is negligible on the plasma membrane. X 17,300. (Inset) A reactive granule at higher magnification. X 45,000.

(b) Platelets incubated with anti-βTG serum and then pA-peroxidase. Reaction product fills some of the granules (arrows), but not others and is absent from the plasma membrane. X 21,000. (c) Platelets incubated with normal rabbit serum and then Fab-peroxidase. The granules and the plasma membrane show no reaction product. X 19,000. (d,e) Platelets fixed in 8% paraformaldehyde and processed for frozen thin section immunocytochemistry. (d) Sections incubated with anti-βTG serum and then with colloidal gold immunoconjugate (GAR-G20). The label (arrows) is present in most of the alpha granules and is negligible on the plasma membrane. X 36,000. (e) Sections incubated with anti-βTG serum and then with colloidal gold immunoconjugate (GAR-G5). The label (arrows) is present in most of the alpha granules (α). X 46,000.

FIGURE 3. Transmission electron micrographs showing the localization of fibrinogen in immediately fixed platelets. (a) Platelets fixed in 8% paraformaldehyde. Frozen thin sections were incubated with anti-fibrinogen serum and then GAR-G20. Note label (arrows) over many of the granules and on the plasma membrane. X 27,000. (b) Platelets fixed in 2% paraformaldehyde. Sections were incubated with anti-fibrinogen serum and then GAR-G5. Note the heavy labeling of the plasma membranes (arrows). X 36,000. (c) Identical to b except that the platelets were processed with 0.1% saponin. The plasma membranes are not as heavily labeled as in b. X 36,000. (d,e) Platelets fixed in 2% paraformaldehyde and processed with immunoperoxidase reagents, but without saponin. (d) Platelets incubated with normal rabbit serum and then pA-peroxidase. The plasma membranes show heavy reaction product (arrows). X 17,300.

(e) Platelets incubated with anti-fibrinogen serum and then Fab-peroxidase. The plasma membranes show heavy reaction product (arrows). X 17,300.

FIGURE 4. (a-d) Transmission electron micrographs showing the localization of proteins in washed platelets fixed in 8% paraformaldehyde and prepared for frozen thin section immunocytochemistry. (a,b) Sections incubated with anti-fibrinogen serum and then with GAR-G5 (a) and GAR-G20 (b). Note the heavy labeling (arrows) of most of the granules and the paucity of label on the plasma membrane. (a), X 48,000; (b), X 54,000. (c,d) Distribution of PF4, fibrinogen, and βTG in double-labeled platelets. (c) Sections exposed to the following sequence of reagents: anti-PF4 serum, GAR-G5, anti-fibrinogen serum, and GAR-G20. Heavy labeling for PF4 and fibrinogen is evident in the granules. Most of the granules are labeled with both GAR-G5 and GAR-G20, although a few are labeled with only one or the other, and some are not labeled at all. X 62,000. (d) Sections exposed to: anti-βTG serum, GAR-G5, anti-fibrinogen serum, and then GAR-G20. X 86,000. (e) Identical to (d) except that immunocytochemical procedures were performed on sections of platelets immediately fixed in 8% paraformaldehyde. The granules are labeled with both GAR-G20 (large arrows) and GAR-G5 (small arrows). X 58,000.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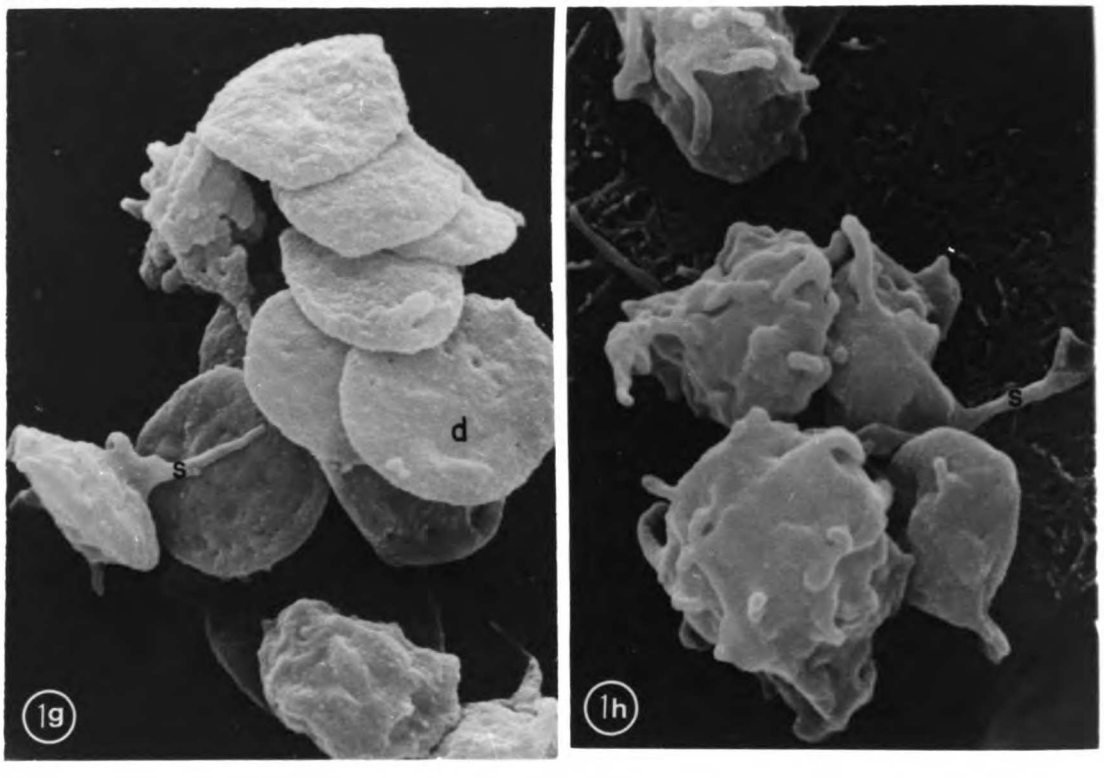
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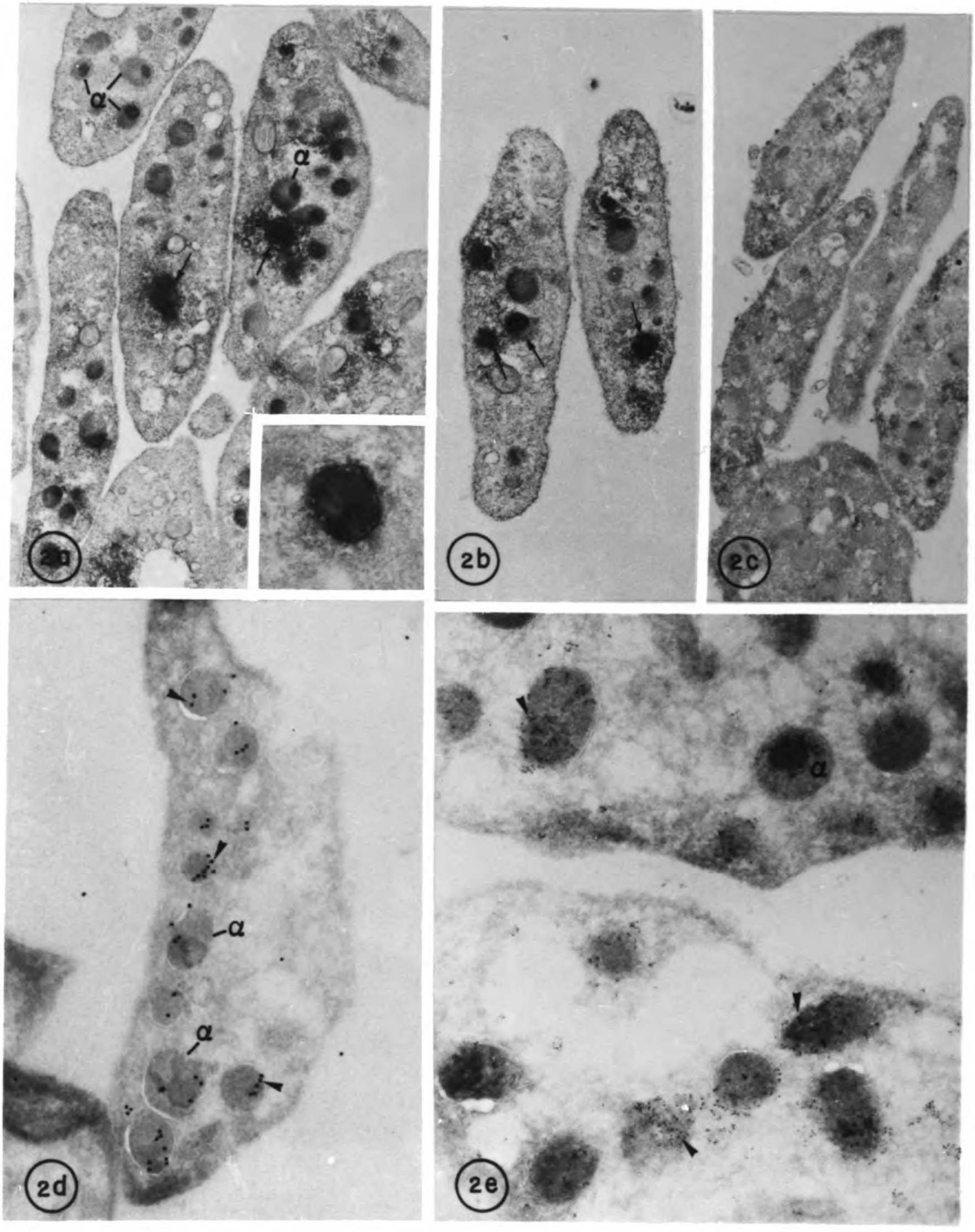
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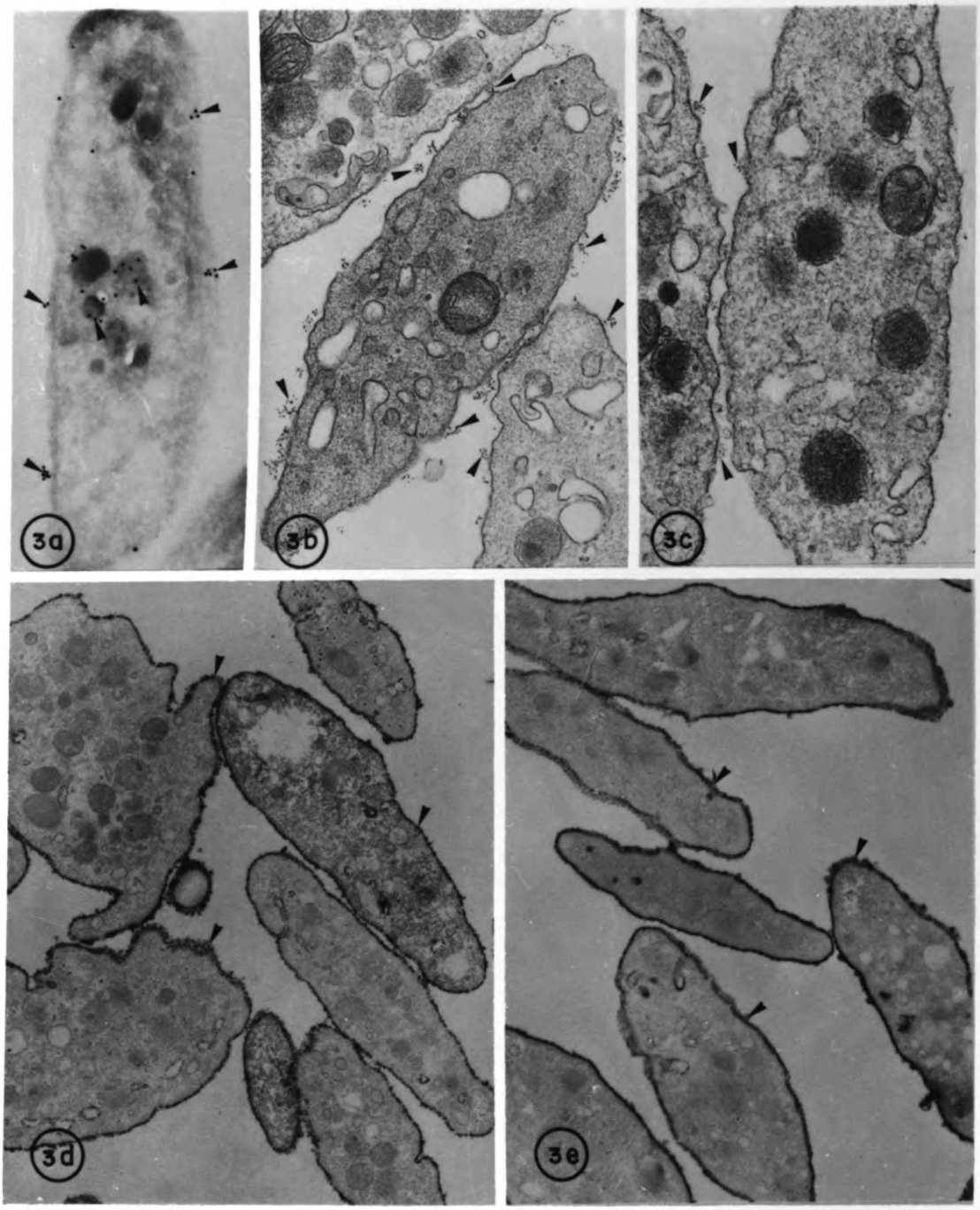
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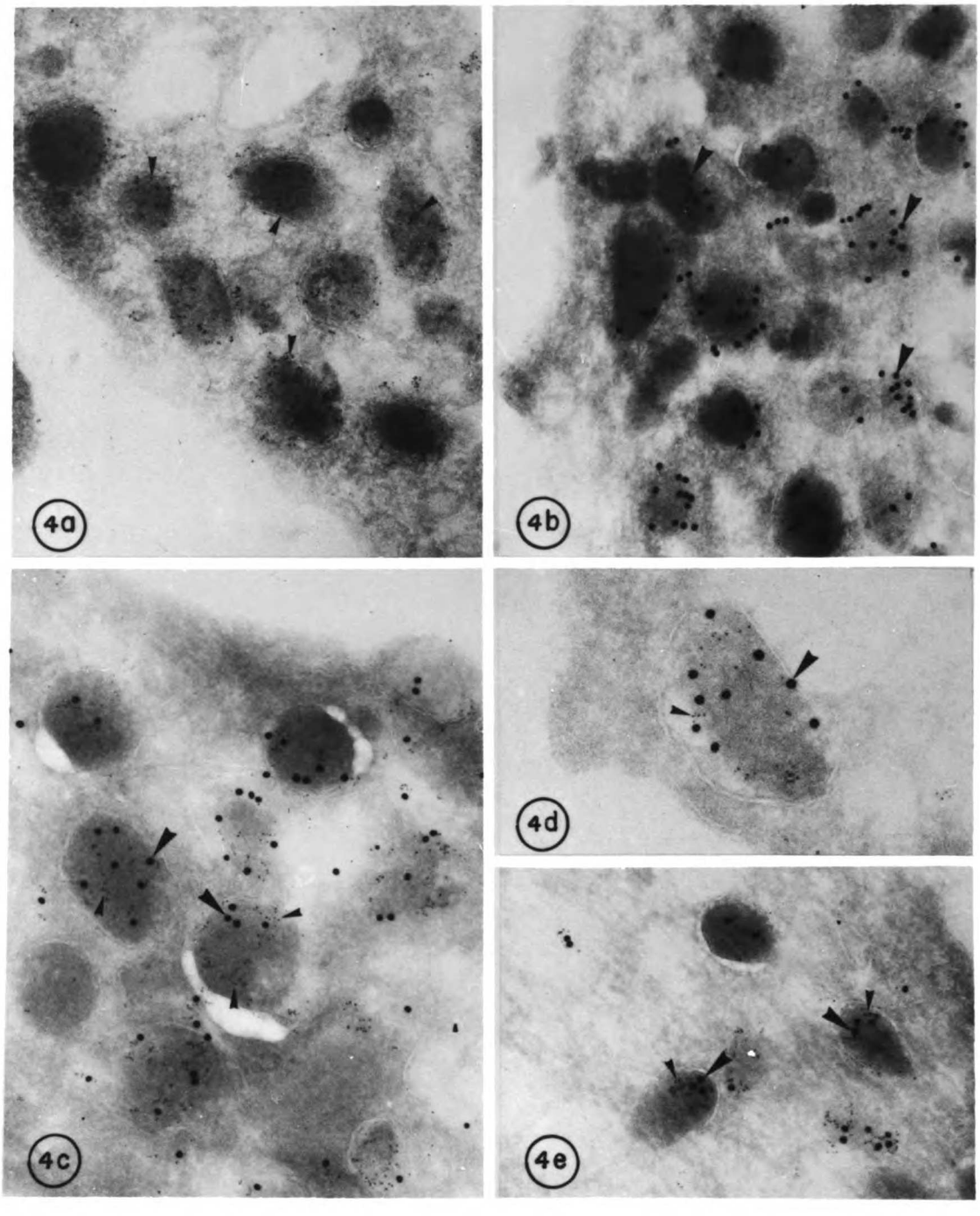
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Chapter 3

REDISTRIBUTION OF ALPHA GRANULES AND THEIR CONTENTS IN THROMBIN-STIMULATED PLATELETS

INTRODUCTION

Platelets are stimulated by a variety of agents, including thrombin, to change in shape, to release several of the proteins stored in their granules, and to form aggregates. In vivo, these aggregates may form a hemostatic plug. The secretion of the contents of alpha granules from stimulated platelets has received increased attention over the past few years. The release of alpha-granule glycoproteins and proteins is an extremely sensitive marker of platelet activation, since they are released at lower concentrations of stimuli than are necessary for the release of dense granule constituents such as serotonin and ADP. Many granule constituents carry out known functions outside the platelet after release. The levels of platelet-specific proteins in plasma are sensitive indicators of secretion from platelets in vivo and can be useful in the evaluation of certain clinical disorders (1). Biochemical studies have shown that several of the substances secreted from platelets to the extracellular medium-thrombospondin, fibrinogen, fibronectin, Factor VIII-von Willebrand factor, and platelet Factor 4 (PF4)-also bind to the platelet plasma membrane, but the functional significance of this binding is unclear (2-7).

The only non-biochemical technique previously used to study release from platelet alpha granules is immunofluorescence. Although the level of resolution attainable with this technique is too low to permit the differentiation of granule populations, immunofluorescence has been used to localize PF4, fibronectin, Factor VIII-related antigen, and Factor V in platelets. Ginsberg et al. (8) showed that the punctate distribution of PF4 in unstimulated platelets changes to a pattern of large, fluores-

cent masses upon stimulation of the platelets with thrombin. These intracytoplasmic masses increase in number with time and migrate to the periphery of the cell, but by the time secretion is 90% complete, few masses are observed. These investigators concluded that the intracytoplasmic masses are formed as a result of multiple fusions of granules and may eventually release their contents by fusing with the plasma membrane.

In the studies described in the accompanying paper, we demonstrated ultrastructurally by several immunocytochemical methods, that PF4, β-thromboglobulin (βTG), and fibrinogen are localized in platelet alpha granules. In the present study, we used the same immunocytochemical methods, along with morphologic techniques, to investigate the ultrastructural localization of PF4, βTG, and fibrinogen in permeabilized and nonpermeabilized thrombin-stimulated platelets. Since thrombin stimulates degranulation in the platelet, we also questioned whether release from platelet alpha granules differs from typical exocytosis, that is, fusion of the granule membrane with the plasma membrane such as that observed in pancreas or in polymorphonuclear leukocytes. This report is concerned exclusively with secretion from alpha granules, and does not address the mechanism of secretion from dense bodies or lysosomes. Our findings provide new information on the reorganization of platelet organelles and proteins during degranulation and secretion.

MATERIALS AND METHODS

Preparation of Thrombin-stimulated Platelets for Morphological, Histochemical, and Immunocytochemical Studies

Washed platelets were prepared at 37°C as described in the accompanying report. Platelets were resuspended in the buffer described previously, in Tyrode's buffer with 2 mM $CaCl_2$, or in Tyrode's buffer with 5 mM EDTA. They were incubated with α-thrombin (gift of John Fenton, New York Department of Health, Albany, N.Y.) at final concentrations of 0.02 to 2.0 U/ml for periods ranging from 5 sec - 5 min. Secretion was stopped by the addition of fixative (2% or 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4; 8% paraformaldehyde in 0.1 M PIPES buffer, pH 7.2; or 1.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4, 1% sucrose). Platelet samples were fixed for 1 h at 37°C.

Tannic Acid Histochemistry. Platelets fixed in glutaraldehyde were incubated with tannic acid (1% tannic acid in 0.05 M Na cacodylate buffer, pH 7.0, no sucrose) for 1 h at room temperature. They were then post-fixed in 1% OsO_4 for 1 h, exposed to uranyl acetate for 1 h, dehydrated and embedded as previously described.

Quick-Freeze, Freeze-fracture and Deep-etch, and Freeze-substitution Procedures. Washed platelets were applied to a small piece of Whatman Filter paper #42 which was glued to an aluminum specimen platform. A copper block in a quick-freeze apparatus (Polaron, Line Lexington, Pa.) was cooled to the temperature of liquid helium by the method of Heuser et al. (9). The platelet suspension was either left unstimulated or injected with 1-5 U/ml of thrombin, and was frozen on the copper block.

Quick-frozen platelets were subsequently processed in one of three ways. Some samples were fractured or fractured and deep-etched on a Balzers 360M freeze-fracture device and then shadowed with platinum and carbon. The replicas were cleaned with hypochlorite, washed in distilled water, and mounted on Formvar-coated copper grids. By a modification of the procedure of Hirokawa and Kirino (10), other samples were freeze-substituted by immersion in 5% OsO_4 in acetone for 4 d at -80°C. At the end of this period, they were slowly warmed to room temperature and then incubated for 1 h in 1% uranyl acetate in absolute ethanol. The samples were incubated twice, for 10 min each time, in propylene oxide and then infiltrated, embedded, and prepared for thin sectioning as described above.

Immunocytochemical Procedures. Washed platelets incubated with thrombin as described above were fixed in 2% or 4% paraformaldehyde and processed with or without saponin by the method described in the accompanying report. Briefly, this consisted of exposing cells to specific primary antisera, followed by incubation with one of three immunoconjugates: a) Fab fragments of sheep anti-rabbit IgG conjugated to peroxidase (Fab-peroxidase) b) protein-A peroxidase conjugate (pA-peroxidase) or c) goat anti-rabbit IgG (GAR) linked to either 5nm-sized or 20nm-sized colloidal gold particle. For frozen thin section procedures, platelets fixed in 8% paraformaldehyde in 0.1 M PIPES buffer, pH 7.0 were washed well in 0.1 M PIPES buffer, pH 7.0, containing 10% (wt/vol) sucrose. They were infiltrated and embedded in 2.3 M sucrose solution, frozen, and stored in liquid nitrogen. Immunocytochemical procedures were performed on sections cut on a Sorvall MT-2 ultramicrotome with a frozen thin section (FTS)/LTC-2 attachment as described in the accompanying report. Single-and

double-label experiments utilized specific primary antisera, or normal rabbit serum or buffer in place of specific antiserum, and goat anti-rabbit IgG conjugated to 5nm-size or 20 nm-sized colloidal gold particles (GAR-G5 or GAR-G20). Control experiments were performed as described in the accompanying report.

RESULTS

The objective of this study was to investigate the distribution of platelet organelles and alpha granule proteins in platelets stimulated with thrombin (0.02 - 5 U/ml) for various periods (2 s to 5 min) before fixation. These platelets were obtained from anticoagulated blood and washed in the presence of an inhibitor of platelet activation. All procedures through fixation were carried out at 37^{o}C.

Morphological Studies of Thrombin-Stimulated Platelets

TANNIC ACID-STAINED PREPARATIONS: To examine platelet degranulation, we used tannic acid to stain the membranes of the platelets in continuity with the external space. This technique also provided better morphologic definition of the plasma membrane than in our immunocytochemical preparations in which the membranes were altered by the saponin used as a permeabilizing agent.

Unstimulated platelets processed with tannic acid had several features well-documented by other investigators: a fairly discoid form, and a dispersed arrangement of organelles. Elements of the surface-connected canalicular system in these platelets were connected to the plasma membrane by narrow necks and often extended deep into the cy-

toplasm (Fig. 1 a). As might be expected, thrombin-stimulated platelets processed with tannic acid had a vastly different appearance. They were round and swollen in form, rather than discoid, and their granules were clustered in the center of the cell, in close apposition with one another and with elements of the SCCS (Fig. 1 b). Occasional images of granule-granule fusion were observed (Inset, Fig. 1 b). Some structures were observed in communication with the extracellular space by their dense staining with tannic acid while others with a luscent appearance were not. After longer intervals of stimulation with thrombin, large intracellular structures, in continuity with the extracellular space, were present both in the center and at the periphery of the platelet (Fig. 1 c). The necks joining these elements of the SCCS to the plasma membrane were considerably wider than in unstimulated cells (Fig. 1 d, e). Granules were sometimes in close proximity to the large intracellular structures, but fusion profiles and pentalaminar formations were rarely observed. We sometimes saw small "dimples" on granules impinging on neighboring granules or elements of the SCCS but we are uncertain of their significance. We more often observed a wide neck between two adjacent structures (Fig. 1 b, inset); through additional fusions, these linked structures may ultimately evolve into large compound granules as seen in Fig. 1 b.

QUICK-FROZEN PREPARATIONS: We employed the additional technique of quick-freezing in the hope of arresting early events during this extremely rapid degranulation process that may not have been readily observed due to the relatively slow fixation by glutaraldehyde.

In thrombin-stimulated platelets that had been quick-frozen and

then either freeze-substituted (Fig. 2 a), fractured (Fig. 2 b), or fractured and deep-etched (Fig. 2 c) we frequently observed large intracellular vacuoles corresponding to those seen in tannic acid-stained preparations.

In unstimulated platelets that had been quick-frozen and then deep-etched, the SCCS sometimes extended into the cytoplasm and was connected to the external surface by narrow necks (Fig. 2 d). Thrombin-stimulated platelets processed in the same way showed granules in close apposition with one another (Fig. 2 c, inset, and with elements of the SCCS (Fig. 2 e); the necks connecting the SCCS to the extracellular space were wider than in unstimulated cells.

Demonstration of βTG, PF4, and Fibrinogen in Thrombin-Stimulated Platelets

SAPONIN-PERMEABILIZED PLATELETS PROCESSED WITH IMMUNOPEROXIDASE CONJUGATES: To study the intracellular localization of βTG, PF4, and fibrinogen in platelets, we permeabilized unstimulated platelets with saponin and then exposed them to various primary antisera and Fab-peroxidase.

In unstimulated platelets processed with anti-fibrinogen (Fig. 3 a), anti-βTG, or anti-PF4 serum, reaction product was localized in the alpha granules, as we had observed in immediately fixed platelets (see accompanying report), and was negligible on the plasma membrane. To investigate the redistribution of these substances after thrombin stimulation, we processed thrombin-stimulated platelets in the same way. The results with antibodies against fibrinogen (Fig. 3, b - d), βTG and PF4 were the same. Large intracellular vacuoles filled with reaction product were sometimes present in the center of the cell, together with a few reactive or unreactive granules. Pools of reaction product were

frequently present near the periphery of the platelet (Fig. 3 b - d), but we saw no images of direct fusion with the plasma membrane. The plasma membrane was devoid of reaction product probably because of the saponin treatment (see accompanying paper). No reaction product was visible on the plasma membranes or in intracellular vacuoles in control preparations incubated with normal rabbit serum rather than the specific primary antiserum (Fig. 3 e).

It should be noted that morphologic preservation was compromised because of the saponin treatment necessary to allow access of reagents. In addition, we frequently observed "bleeding" of peroxidase reaction product into the platelet cytoplasm, as a result of both weak fixation and saponin treatment. Increasing the concentration of paraformaldehyde in the fixative to 8% yielded better morphologic detail, but fewer reactive granules, although the reactive granules were intensely stained.

Localization of βTG, PF4, and Fibrinogen on the Plasma Membranes of Non-permeabilized Platelets

Because biochemical studies have demonstrated that some of the substances secreted from platelet granules bind to the platelet plasma membrane (2-7, 23), we examined the extracellular localization of βTG, PF4, and fibrinogen after thrombin stimulation in nonpermeabilized platelets.

PLATELETS PROCESSED WITH IMMUNOPEROXIDASE CONJUGATE: Because we previously found that Fab-peroxidase bound nonspecifically to the plasma membrane, (see accompanying report), we used pA-peroxidase to study the localization of βTG, PF4, and fibrinogen on the plasma membranes of unstimulated and thrombin-stimulated platelets. In samples incubated with anti-

fibrinogen serum and then pA-peroxidase, the plasma membranes of unstimulated platelets were almost free of reaction product (Fig. 4 a), whereas the plasma membranes of thrombin-stimulated cells were densely labeled (Fig. 4 b). This was also true for PF4 and βTG, although the labeling was less dense for βTG than for fibrinogen or PF4. In control samples incubated with normal rabbit serum and then pA-peroxidase after fixation, the plasma membranes were free of label (Fig. 4 c).

PLATELETS PROCESSED WITH COLLOIDAL GOLD IMMUNOCONJUGATES: Unstimulated platelets incubated with anti-fibrinogen serum were free of IgG-gold label (Fig. 4 d), whereas thrombin-stimulated platelets incubated with the same antisera had discrete clumps of IgG-gold label around the entire plasma membrane (Figs. 4 e and 4 f). As in platelets processed with immunoperoxidase conjugate, the labeling was frequently heaviest where platelets were closely apposed. The pattern of labeling was the same for βTG and PF4 although the labeling was least dense for βTG. The heaviest label for all antigens was seen on platelets stimulated in Tyrode's buffer with 2mM $CaCl_2$. Little or no label was observed for all antigens on platelets stimulated with thrombin in Tyrode's buffer with 5 mM EDTA.

FROZEN THIN SECTIONS PROCESSED WITH COLLOIDAL GOLD IMMUNOCONJUGATES: Although a less sensitive procedure than our other immunocytochemical techniques, the use of colloidal gold immunoconjugates on frozen thin sections allowed us to examine the intracellular and extracellular distribution of proteins secreted from alpha granules. Furthermore, it permitted us to circumvent the permeation and "bleeding" problems in saponin-treated preparations and to use a different second marker. In thrombin-stimulated platelets processed with colloidal gold immuno-

conjugate, fibrinogen (Fig. 5 a), βTG, and PF4 were localized in large intracellular vacuoles. The labeling was frequently heaviest along the membranes of these structures; granules were sometimes labeled. These large vacuoles resembled those seen using other morphological and immunocytochemical procedures by White (11) and Ginsberg (8). Double-label experiments with gold probes of two sizes demonstrated the simultaneous presence of βTG and fibrinogen (Fig. 5 b), PF4 and fibrinogen (not shown) and βTG and PF4 (not shown), within the larger vacuoles. The results were the same for all sequences of antisera and gold probes used. The extent of the plasma membrane was labeled with both sizes of gold probes (Fig. 5 b); such labeling was observed for fibrinogen, βTG, and PF4. Control preparations in which non-immune purified rabbit IgG was used in place of primary antiserum showed no specific labeling of the large vacuoles, although scattered gold particles adhered nonspecifically to tissue sections.

Discussion

The localization of β-thromboglobulin, platelet Factor 4, and fibrinogen in the alpha granules of the resting platelet was established in the accompanying paper. In the present investigation, we have traced the pathway by which these proteins are secreted from the platelet after stimulation with thrombin. Staining with tannic acid and quick-freeze techniques revealed that thrombin stimulation resulted in various morphologic changes in the platelet, including fusion of the alpha granules with one another and with elements of the SCCS to form large, surface-connected structures. Immunocytochemical labeling of βTG, PF4, and fibrinogen showed that thrombin stimulation caused them to be redistributed from the alpha granules to large intracytoplasmic vacuoles and to the platelet plasma membrane. These vacuoles ultimately fused with the SCCS and were connected to the extracellular space by wide necks, presumably formed by enlargement of the narrow necks connecting the SCCS to the surface of the unstimulated cell. Since fusion of the alpha granules or the large vacuoles with the plasma membrane was rarely seen using these techniques, we conclude that these substances gained access to the external milieu through these wide necks.

These morphologic and immunocytochemical observations suggest the following pattern of secretion from the platelet alpha granules after thrombin stimulation (Fig. 6). The normally discoid platelet first becomes round and filopodia develop after stimulation with thrombin. Concomitantly, the alpha granules and other organelles move to the center of the platelet and are surrounded by a ring of microtubules. Granules fuse with other granules and with elements of the SCCS to form

large vacuoles, situated near the center and periphery of the platelet, and containing alpha-granule proteins. As these vacuoles form in the cytoplasm, the necks connecting the SCCS to the extracellular space become wider. The enlargement of these openings in the plasma membrane permits the rapid secretion of alpha granule proteins to the exterior of the platelet and binding of some of this secreted protein to the plasma membrane, where they carry out their various physiological functions.

This summary is consistent with other ultrastructural findings on platelet degranulation. The localization of various electron dense tracers (lanthanum nitrate, ruthenium red, horseradish peroxidase, thorium dioxide) in stimulated platelets suggested to White that the SCCS was a probable pathway for release of platelet products (12-14). Studies of uptake and release of polylysine and polybrene by platelet granules (15), as well as studies of platelets treated with phorbol myristate acetate (16) and cytochalasin B and trypsin (17), led him to conclude that degranulation occurred through fusion of the secretory organelles with elements of the SCCS. Our data supports this hypothesis. In studies of thrombin-stimulated platelets, Droller (18, 19) postulated that an electron-dense, fibrous material observed within an intracytoplasmic "lamellar-vacuolar network" was fibrinogen that had been secreted from the alpha granules. Holme et al (20) proposed that fibrils observed in the SCCS of thrombin-stimulated platelets originated from fibrinogen, because they were not present when the platelets had been incubated with iodoacetic acid and antimycin before stimulation. These studies support our findings that platelet fibrinogen is secreted to the extracellular space via the SCCS. Zucker-Franklin (21) has demonstrated by freeze-

fracture and electron microscopic techniques two pathways for platelet endocytosis: membrane pits, which endocytose small particles and solutes; and membrane invaginations, which endocytose large particles. She has speculated that these pathways may also be involved in secretion. The membrane invaginations may correspond to distended elements of the SCCS. Finally, using immunofluorescence techniques on detergent-permeabilized platelets, Ginsberg and colleagues (8) showed that PF4 had a granular distribution in the resting cell, but was distributed in large masses near the periphery of the cell after thrombin stimulation. Although these observations are consistent with a redistribution of PF4 from the alpha granules to large vacuoles, these investigators have suggested that such vacuoles are membrane-bounded structures that either are in a canalicular system which is functionally closed at times (8) or that release their contents by fusing with the plasma membrane (8, 22). The data presented in this paper supports the former interpretation of their data.

Our finding that at least some βTG, PF4 and fibrinogen bind to the surface of the platelet after thrombin-induced secretion from the cell is supported by biochemical studies (2-7, 23) showing such binding of thrombospondin, fibrinogen, fibronectin, Factor VIII-von Willebrand Factor and PF4. Other biochemical studies have shown that stimulated platelet membranes bear specific receptors for fibrinogen, fibronectin, and Factor VIII-related antigen (24-28) and that membrane-specific binding of fibrinogen is Ca^{++}-dependent (26). The binding of secreted fibrinogen in the presence of Ca^{++} to the plasma membrane observed by our procedures is probably specific, since little label was observed in preparations which included EDTA. Whether the various alpha-granule proteins

bind to the plasma membrane in different proportions after secretion is not known. It is possible that only a small portion of the total secreted protein binds to the plasma membrane. George and Onofre (7) suggest that the binding, which localizes and concentrates the secreted substances on the platelet surface, may facilitate platelet functions such as adhesion and aggregation.

These data on the redistribution of secreted βTG and PF4 to the platelet plasma membrane differ from our previous report (29) in which we detected reaction product with anti-PF4 antiserum on the surface of thrombin-stimulated platelets but none with anti-βTG antiserum. This discrepancy points to the necessity of careful examination of platelet aggregates, parts of which may be virtually inaccessible to immunocytochemical reagents due to the close juxtaposition of cells.

We have traced the pathway by which three proteins are secreted from the alpha granules of the platelet after thrombin stimulation. Whether other alpha granule proteins are secreted by this pathway, and whether stimuli other than thrombin induce secretion by this pathway, remain to be determined. It is also unclear whether the other platelet granule populations -- dense bodies, lysosomes, and peroxisomes -- release their contents through a similar pathway, or indeed, if peroxisomes release their contents at all. The kinetics of secretion vary from one granule population to another. Dense body and alpha granule constituents appear to be released at the same time, while lysosomal contents are released after alpha granule and dense body secretion. Alpha granule proteins are secreted at lower concentrations of stimuli such as thrombin than are dense body and lysosomal constituents (30-32). Lastly, thrombin-stimulated

platelets release different amounts of the various acid hydrolases stored in their lysosomes: equal amounts of β-N-acetylglucosaminidase, β-galactosidase, and cathepsin; smaller, but equal, amounts of β-glucuronidase and aryl sulfatase; and no acid phosphatase (33). Holmsen and Day (34) have suggested that these substances may be contained in different granules or that some proteins may be bound more strongly in the same granule than others, and therefore not released to the same extent. It is clear that more work is needed to determine the secretory pathways of these other granule populations.

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FIGURE LEGENDS

FIGURE 1. Transmission electron micrographs of platelets processed with tannic acid stain to elucidate platelet degranulation after short intervals of thrombin stimulation. Tannic acid densely stains the membranes of the platelet in continuity with the extracellular space. (a) Platelet from an unstimulated sample. Alpha granules (α) and mitochondria (m) are dispersed throughout the cytoplasm. The SCCS, densely stained by tannic acid, is connected to the plasma membrane by narrow necks (arrows). X 34,000. (b - d) Platelets from samples stimulated with 1 U/ml of thrombin for various intervals (5-30 s) before fixation. (b) Alpha granules (1) are clustered in the center of the cell and are surrounded by a ring of microtubules (mt). Some of the granules appear to be fusing (2, inset). The fused granules form large intracellular vacuoles of varying densities, some of which are not yet in contact with the external space (3), and some of which are (4). X 34,000. Inset, X 96,000. (c) Large structures containing tannic acid are present in the cytoplasm, both in the center of the cell (large arrows) and near the periphery (small arrow). X 21,000. (d and e) The large tannic acid-filled structures are in continuity with the extracellular space. The necks connecting these elements of the SCCS to the extracellular space (arrows) are considerably wider than those in a. X 34,000. All samples were fixed in glutaraldehyde, stained with tannic acid, and post-fixed in OsO_4. Sections were stained on grid with uranyl acetate and alkaline lead citrate.

FIGURE 2. Transmission electron micrographs of thrombin-stimulated, quick-frozen platelets. All samples but that shown in d were exposed to 1-5 U/ml of thrombin for 2 s before being frozen. (a) Freeze-substituted sample. A granule (g) is fusing with a large vacuolar structure, which corresponds to the structure labeled 3 in Fig 1 b. X 80,000. (b) Fractured sample. Granules are clustered around the large SCCS, which opens to the external space (large arrow). Two areas cleared of intramembranous particles on the E face of the SCCS (small arrows) may represent granules that have come into close apposition with the SCCS preparatory to fusing with this structure. X 80,000. (c-e). Fractured and deep-etched samples. Two granules (arrows) are in continuity with a large vacuolar structure. The inset shows two granules in close proximity to one another. X 80,000. Inset, X 85,000. (d) Unstimulated sample. The SCCS forms an invagination into the cytoplasm from a narrow opening on the plasma membrane (arrow), corresponding to the openings seen in Fig. 1 a. X 62,000. (e) A granule (g) is in close apposition with the SCCS, which extends deep into the cytoplasm and which is joined to the plasma membrane by a wide neck (arrow), corresponding to the necks seen in Fig. 1 d, and e. X 62,000. g, granule, SCCS, surface-connected canalicular system.

FIGURE 3. Transmission electron micrographs showing the localization of fibrinogen in fixed, permeabilized platelets before (a) and after (b-d) thrombin stimulation to illustrate the redistribution of alpha-granule contents. (a-d) Washed platelets incubated with anti-fibrinogen serum and then Fab-peroxidase. (a) Unstimulated platelets. Note the dense reaction product uniformly filling many of the alpha granules (arrows).

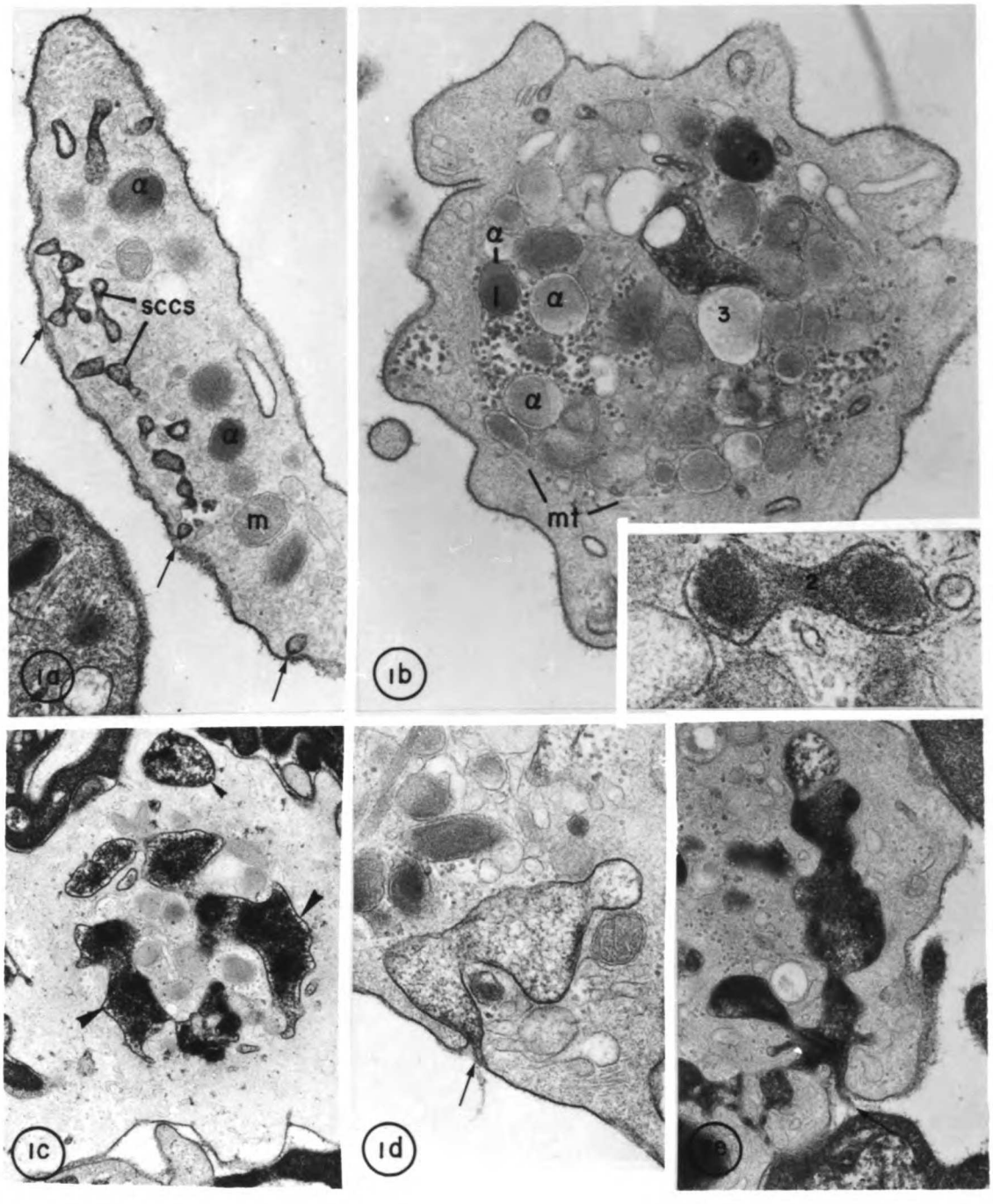
Some of the granules are less reactive than others, and some are negative. X 16,000. (b-d) Platelets incubated with thrombin (0.2-1 U/ml) for various periods (5-30 s) before fixation. Note the heavily reactive intracellular structures in the cytoplasm (arrows), both near the centers of the cells and near the peripheries. Some of the granules (g) are not reactive. Note that the plasma membrane is not labeled. (b) X 16,000; (c) X 19,500; (d) X 29,000. (e) Control preparation processed as in b-d, except that normal rabbit serum was used in place of anti-fibrinogen serum. The intracellular vacuoles (arrows) do not contain reaction product. X 21,000. Unstimulated platelets were fixed in 2% paraformaldehyde and permeabilized with 0.05% saponin; thrombin-stimulated platelets were fixed in 4% paraformaldehyde and permeabilized with 0.1% saponin.

FIGURE 4. Transmission electron micrographs of unstimulated and thrombin-stimulated platelets prepared without saponin and examined immunocytochemically for the extracellular distribution of fibrinogen. (a) Unstimulated platelets exposed to anti-fibrinogen serum and then pA-peroxidase. Note that the membranes of these cells are relatively free of peroxidase label. X 17,300. (b) Platelet aggregate from a thrombin-stimulated sample incubated with anti-fibrinogen serum and pA-peroxidase. The dense reaction product that is present along most of the platelet plasma membranes (arrows) is particularly heavy where two platelets are in close apposition. None of the granules are reactive because the cells have not been permeabilized. X 17,300. (c) Control preparation. Part of a platelet aggregate from the same sample as b. Cells were

incubated with normal rabbit serum and then pA-peroxidase. The plasma membranes are free of peroxidase label. X 21,800. (d)-Unstimulated platelets exposed to anti-fibrinogen serum and then GAR-G5. The plasma membranes are free of gold label. X 30,000. (e) Platelet aggregate from the same sample as b. Cells were incubated with anti-fibrinogen serum and then GAR-G5. The plasma membrane is labeled along its entire extent (arrows). X 30,000. (f) Higher magnification of the sample shown in e. The gold particles are distributed in discrete groups along the extent of the platelet plasma membrane. X 72,000. Unstimulated platelets and thrombin-stimulated platelets (1 U/ml for 5 min) were fixed in 2% paraformaldehyde.

FIGURE 5. Transmission electron micrographs showing the effect of thrombin on the distribution of alpha granule proteins in platelets as revealed by frozen-thin-section immunocytochemistry. (a) Sections exposed to anti-fibrinogen serum and then GAR-G5. IgG-gold label in large intracellular vacuoles (IV) and along the plasma membrane (arrow) demonstrates the localization of fibrinogen. Reactive granules (g) are also seen. X 44,000. (b) Section exposed to the following sequence of reagents: 1) anti-βTG serum; 2) GAR-G5 3) anti-fibrinogen serum; 4) GAR-G20. Heavy gold label is present in large intracellular vacuoles (IV) and along the entire extent of the plasma membrane (arrows). Weak label is present in some alpha granules (α) in this field. X 42,000. Platelets were incubated with thrombin (1 U/ml) for 30 s to 5 min before fixation in 8% paraformaldehyde and subsequent processing.

FIGURE 6. A diagrammatic summary of the sequence of events after a platelet is stimulated by thrombin to secrete its alpha granule contents. (a) The unstimulated platelet is typically discoid, with alpha granules (α) and mitochondria (M) randomly distributed throughout the cytoplasm. Microtubules (mt) are often present. Elements of the surface-connected canalicular system (SCCS) are connected to the external space by narrow necks. Platelet alpha-granule proteins are present in the granules (indicated by stippled areas), and not in the SCCS. (b) Within 5 s after stimulation begins, the platelet becomes more rounded and swollen in form, and filopodia develop. Granules are clustered in the center of the cell, closely apposed to one another and to elements of the SCCS, and are surrounded by a ring of microtubules and microfilaments. (c) Concomitant with the changes illustrated in b, granules begin to fuse with each other and with elements of the SCCS. The resultant structures contain alpha-granule proteins. (d) Between 5 and 30 s after stimulation begins, large intracellular structures containing alpha-granule proteins are observed in the center and periphery of the platelet cytoplasm. The necks connecting these structures to the external space are considerably wider than in unstimulated cells. At 1-5 min, few granules remain in the platelet cytoplasm, and alpha-granule proteins are situated in intracellular vacuoles and along the extent of the plasma membrane. Many platelet aggregates have been formed by this time.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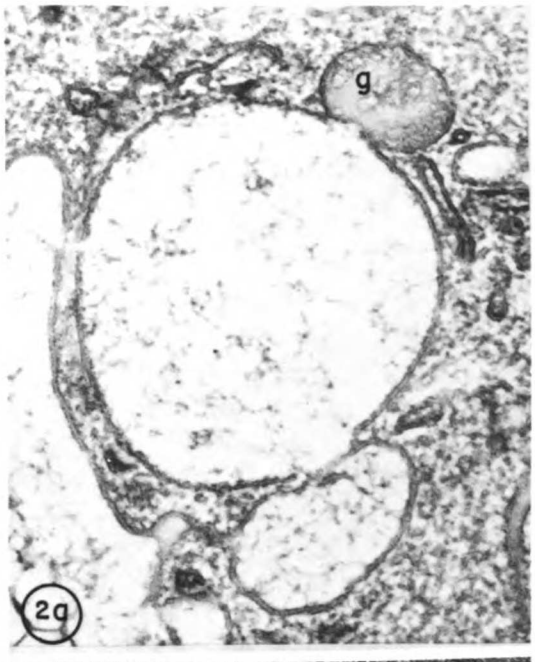
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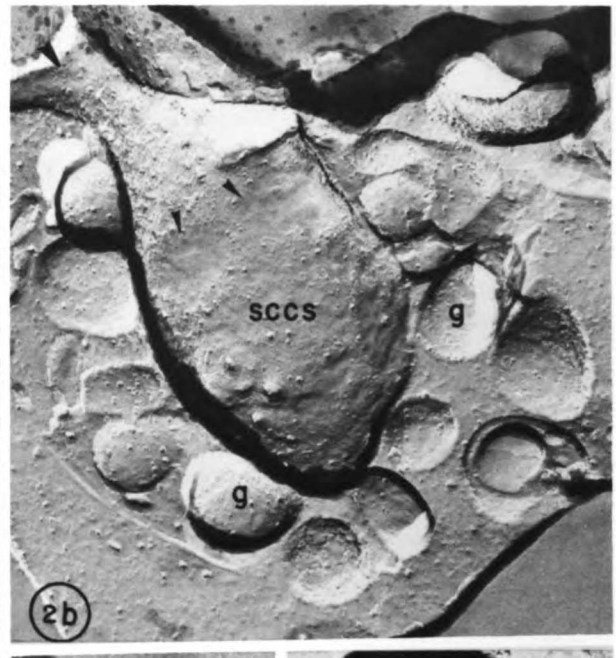
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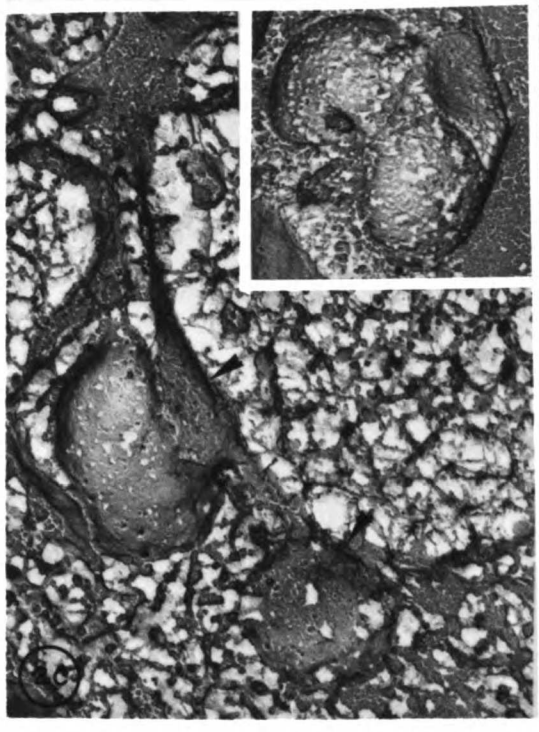
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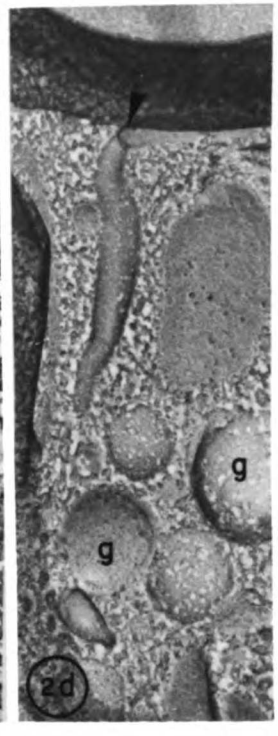
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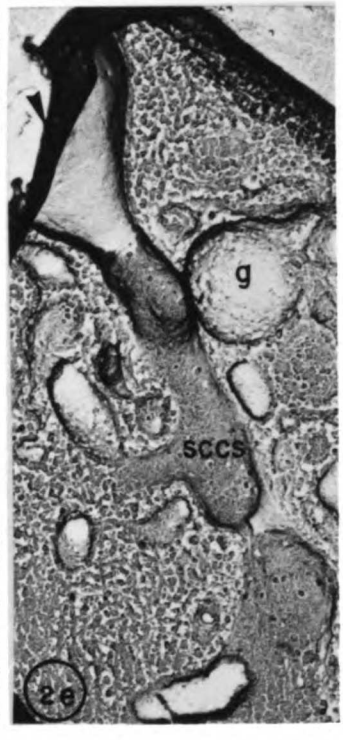
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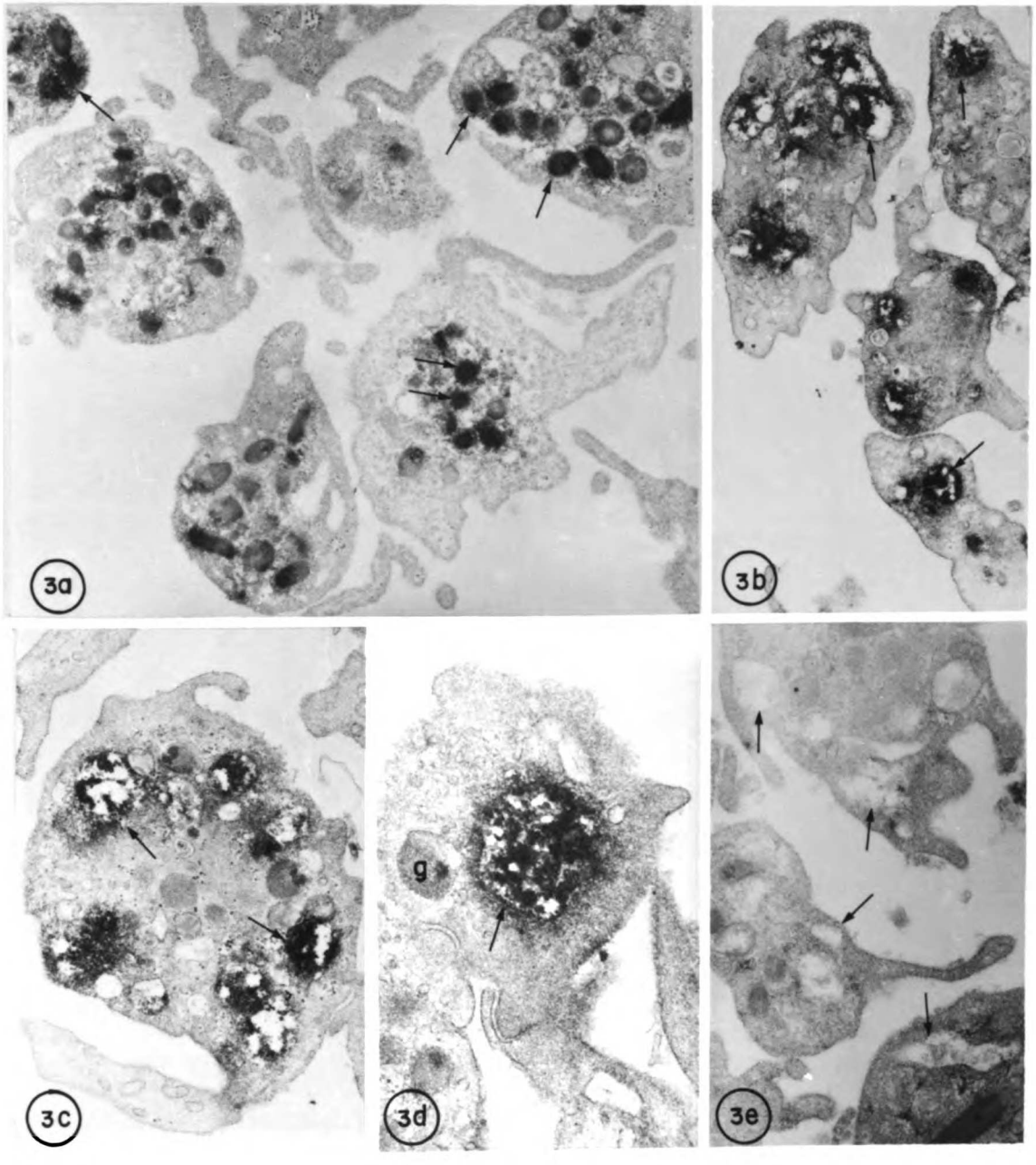
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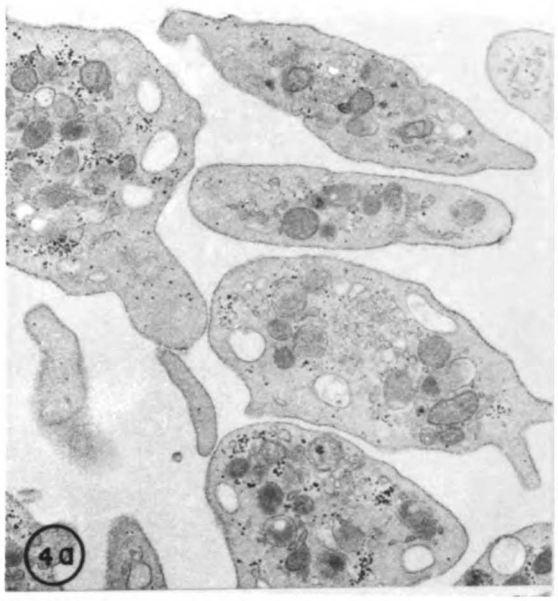
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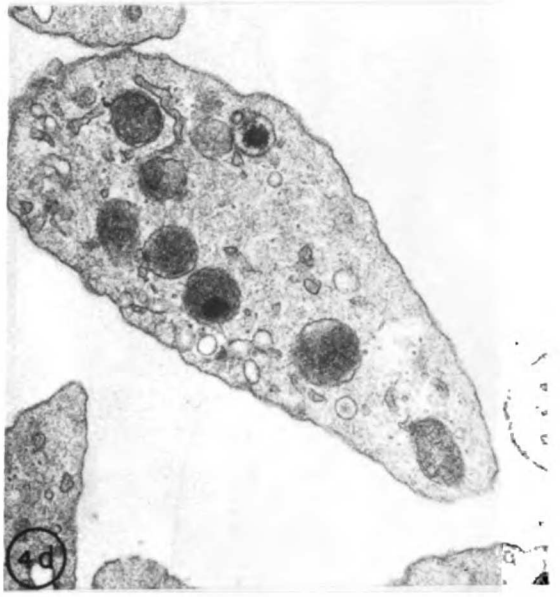
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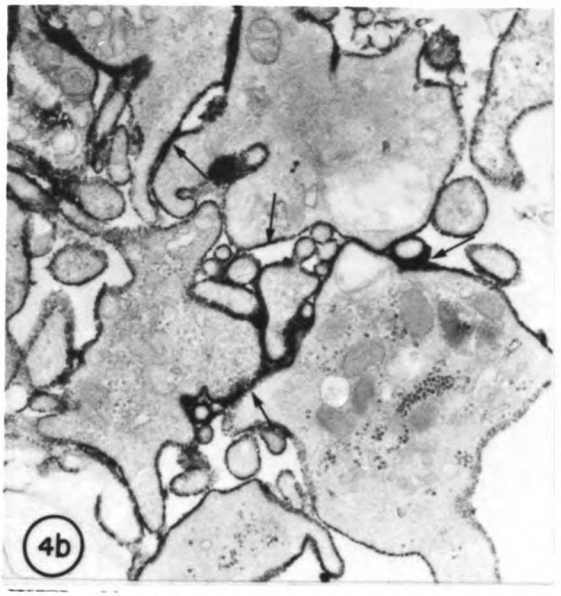
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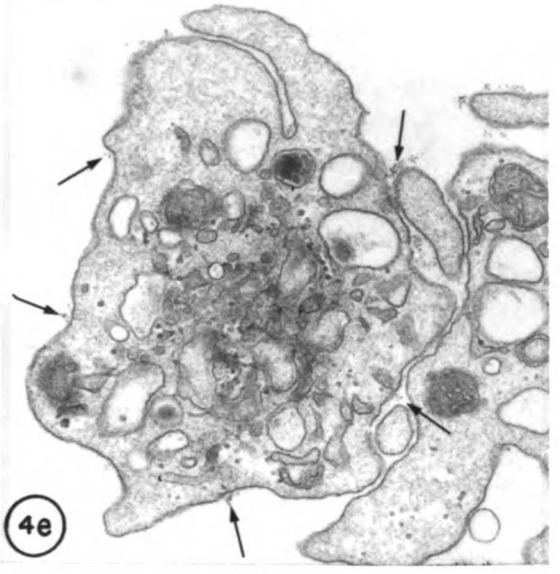
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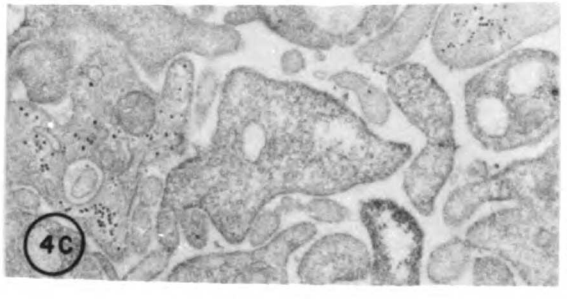
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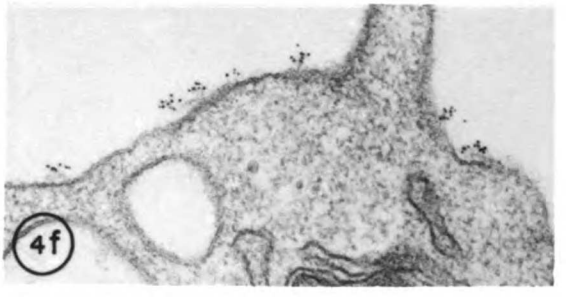
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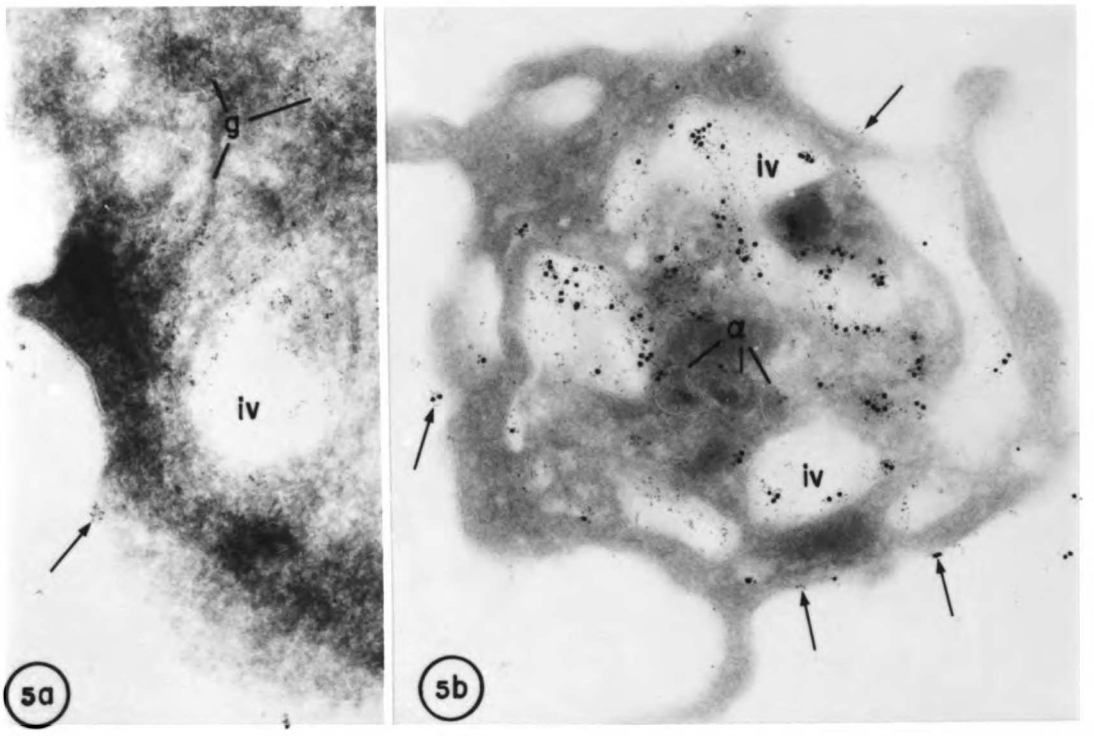
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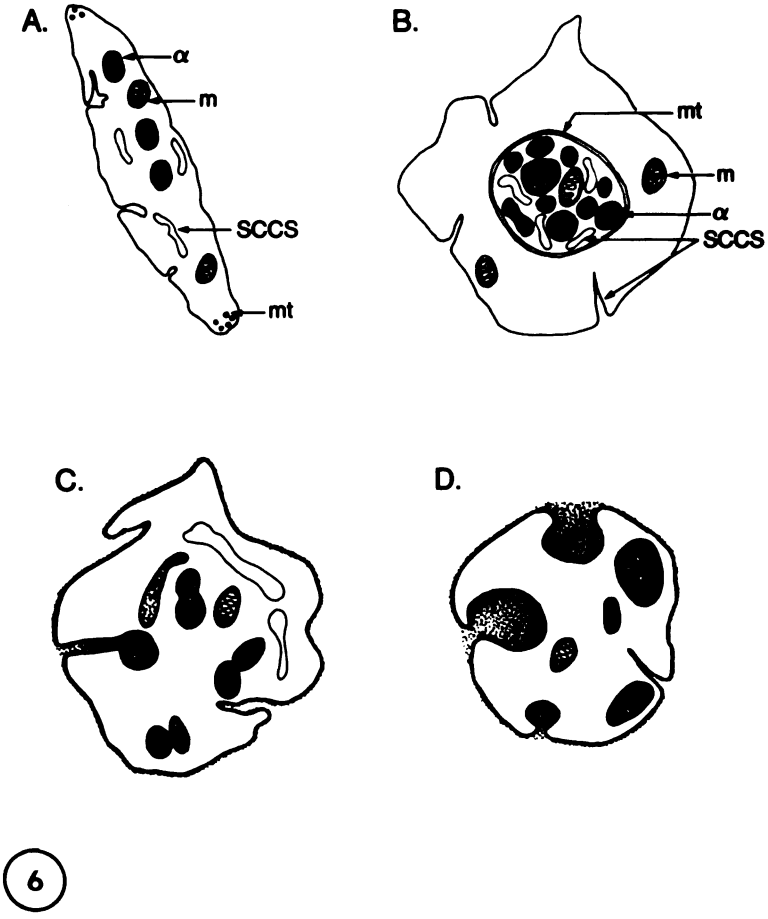

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Chapter 4

IMMUNOCYTOCHEMICAL DEMONSTRATION OF ALPHA GRANULE PROTEINS IN HUMAN MEGAKARYOCYTES

INTRODUCTION

The contents of platelet granules are secreted to the external milieu during platelet degranulation where they perform their various functions (1). Since platelets are anucleate and possess only the vestiges of a protein synthetic apparatus, it has been conjectured that most platelet constituents are synthesized in the megakaryocyte. Some platelet substances have been found in the megakaryocyte. Platelet-derived growth factor activity has been detected in guinea pig and human megakaryocytes (2, 3) and Factor VIII-related antigen has been found by immunoferritin techniques in human megakaryocytes in structures resembling the platelet surface-connected canalicular system (4). Immunoperoxidase and immunofluorescence techniques on smears of human bone marrow have been used to localize PF4 (5, 6) and βTG (6) in human megakaryocytes. Recently, Factor VIII-related antigen and fibrinogen were demonstrated by immunofluorescence methods in the cytoplasm of rat promegakaryoblasts (7). Rabellino et al., (8, 9) detected the presence of Factor VIII-related antigen, fibrinogen, fibronectin, platelet Factor 4, and several membrane glycoproteins in human megakaryocytes in megakaryocyte-enriched preparations by immunofluorescence. Most recently, Ryo, et al. (10), have detected PF4 synthesis in a megakaryocyte enriched rabbit bone marrow culture system. This data is important because not all substances found in platelets are synthesized by megakaryocytes; an example of this is serotonin, or 5-hydroxytryptamine (5-HT). Tranzer et al. (11), showed by electron microscopy and a spectrofluorometric method that guinea pig and rabbit megakaryocytes were capable of storing 5-HT in organelles resembling the 5-HT storage granules in blood platelets of

the same species. Fedorko demonstrated, using autoradiography, that guinea pig megakaryocytes were able to take up and concentrate ^{3}H-serotonin (12); similar conclusions were drawn by Seitz in studies on rat and guinea pig megakaryocytes (13). The questions of whether or not unidentified organelles exist in megakaryocytes which serve as precursors for platelet dense bodies and what the storage mechanism of 5-HT in these cells is, remain to be elucidated.

Techniques utilized to localize platelet and megakaryocyte granule substances have been limited, as discussed previously. For example, immunofluorescence permits one to answer the question of whether or not a substance is localized to a certain cell, but does not allow identification of the substances within specific organelles. Furthermore, demonstrating the presence of substances in the megakaryocyte does not distinguish between synthesis or endocytosis by the cells. Using techniques and probes developed with platelets at the fine structural level, I asked the question whether β-thromboglobulin, and platelet Factor 4 are made in the megakaryocyte. Preliminary evidence showed that these proteins are contained in megakaryocyte granules and I have occasionally detected βTG in some elements of the protein synthetic apparatus.

MATERIALS AND METHODS

Acquisition of human bone marrow specimens and their preparation for plastic embedding. Human bone marrow specimens were obtained from three paid normal volunteers by Jamshidi needle (11-gauge) biopsy of the posterior iliac crest. (The volunteers' consent forms were approved by the UCSF Committee on Human Research.) The specimens were immediately placed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 and fixed for 5 hr at 4^{o}C. They were then transferred to 0.1 M phosphate buffer containing 3% sucrose. After 1 hr, the specimens were dehydrated in graded concentrations of acetone, infiltrated, and embedded in glycol and methyl methacrylate monomers (Polysciences Inc., Warrington, PA) according to the method of Beckstead et al., (14, 15). This technique enabled us to fix intact cells, thereby avoiding the distortion caused by air drying. It also allowed us to examine thin sections of tissue by light microscopy with increased resolution of the intracellular staining of platelet and megakaryocyte organelles. These procedures were carried out at 4^{o}C. The hardened blocks were allowed to warm to room temperature and were sectioned at 2 μm with a Sorvall JB-4 microtome.

Light microscope level immunocytochemical procedures. To block endogeneous peroxidase, all tissue sections were incubated for 45 min at room temperature in methanol containing 3.0% hydrogen peroxide and then washed well in phosphate buffered saline (PBS). Control procedures included a check for the complete inhibition of endogeneous peroxidase. The sections were then incubated for 15 min at room temperature with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and washed

several times in PBS. They were subsequently incubated overnight at 4°C with PBS or various dilutions of serum (either anti-βTG, anti-PF4, or normal rabbit serum), washed twice in PBS, incubated at room temperature for 2 hr with protein A-peroxidase conjugate (16) (Zymed Labs, Burlingame, Calif.) (1:20), and washed twice in PBS. The sections were then exposed to diaminobenzidine (0.6 mg/ml) and hydrogen peroxide (0.01% for 5 min at pH 7.6, washed in PBS, counterstained with hematoxylin, air-dried, and mounted in Permount).

Electron microscope level immunocytochemical procedures. Immunocytochemical procedures on bone marrow samples were performed as described previously for platelet preparations using saponin (0.02 - 0.1%) as a permeabilizing agent.

RESULTS

Light microscopic immunocytochemical localization of βTG and PF4 in human megakaryocytes. In sections of plastic-embedded human bone marrow incubated with antiserum against βTG or PF4, peroxidase label was distributed in a granular pattern throughout the cytoplasm of maturing and mature human megakaryocytes (Figures 1 a, c, d). The intensity of the reaction varied from one megakaryocyte to another, immature megakaryocytes were rarely reactive, and rare mature megakaryocytes were negative. The only other reactive bone marrow cells were rare macrophages containing reaction product in large vacuoles perhaps reflecting the phagocytosis of megakaryocyte nuclei and cytoplasmic remnants. In sections of plastic-embedded human bone marrow incubated with normal rabbit serum or buffer instead of specific antiserum, all cells failed to label with peroxidase including human megakaryocytes (Fig. 1 b). Complete inhibition of endogeneous peroxidase was demonstrated by incubating sections that had been processed in inhibition medium, but not exposed to antiserum, with diaminobenzidine and hydrogen peroxide. No cells in these sections showed reaction product. Bone marrow cells other than megakaryocytes were free of peroxidase label in all control and test preparations.

Electron microscopic immunocytochemical localization of βTG and PF4 in human megakaryocytes. In megakaryocytes from bone marrow samples fixed and then permeabilized with saponin, βTG (Fig. 2 d) and PF4 (Fig. 2 e), were localized to some alpha granules. Many granules were unreactive. An occasional Golgi element was labeled (Fig. 2 b and c), although usually only one cisterna in a stack was reactive. Control bone marrow

samples exposed to normal rabbit serum or buffer in place of specific primary antiserum did not contain reaction product in either granules or elements of the protein synthetic apparatus (Fig. 2 a).

DISCUSSION

Using immunoperoxidase procedures at the light and electron microscope levels, we have demonstrated that megakaryocytes contain two platelet-specific proteins -- β-thromboglobulin and platelet Factor 4. Since Kaplan et al., (17) have shown biochemically that βTG and PF4 are present in the alpha granule fraction of platelets and since megakaryocytes contain numerous alpha granules (18-20), we interpreted the granular distribution of reaction product for these proteins we observed in megakaryocytes at the light microscope level to reflect their presence in alpha granules. This was confirmed by our ultrastructural findings, which provide the first convincing evidence at the fine structural level that βTG, PF4, and fibrinogen are localized in some megakaryocyte alpha granules. Not all granules were reactive for these substances. This may indicate either heterogeneity of these granules or that we have been unable to label all of them due to technical limitations.

Although the fate of megakaryocyte alpha granules is clear, their origin is less well established. Morphologic studies on osmium-fixed tissue led Jones to propose that they originate from the Golgi complex (21). It remains unclear which organelles are involved in synthesis and processing of these proteins, and what the temporal relationship between synthesis and the ultimate fate of these substances is. In studying the step-wise assembly of megakaryocyte organelles, it was previously demonstrated in our laboratory that lysosomal enzymes are detectable in small vesicles (primary lysosomes) in the earliest recognizable cell of that series, even before alpha granules appear. A few alpha granules may also be seen in immature megakaryocytes, but are prominent only in the

later stages of differentiation (22, 23). Therefore, our observations that few immature megakaryocytes reacted with antiserum against βTG or PF4 at the light microscope level are consistent with the distribution of alpha granules in megakaryocytes. The stages of megakaryocyte differentiation at which each of these substances is synthesized, however, is unknown.

The utility of specific markers for the platelet-megakaryocyte lineage has been amply demonstrated by Breton-Gorius et al., who have used platelet peroxidase, an entity distinct from granulocytic peroxidases (24), to identify various clinical disorders (25, 26). These investigators have also examined histochemically conditions in which there appears to be defective alpha granule production (27, 28). Whether there is defective protein synthesis, packaging, or storage of these megakaryocyte substances in these conditions remains to be elucidated. I intend to utilize specific probes for alpha granule substances which have been described in detail in this dissertation to investigate similar clinical disorders.

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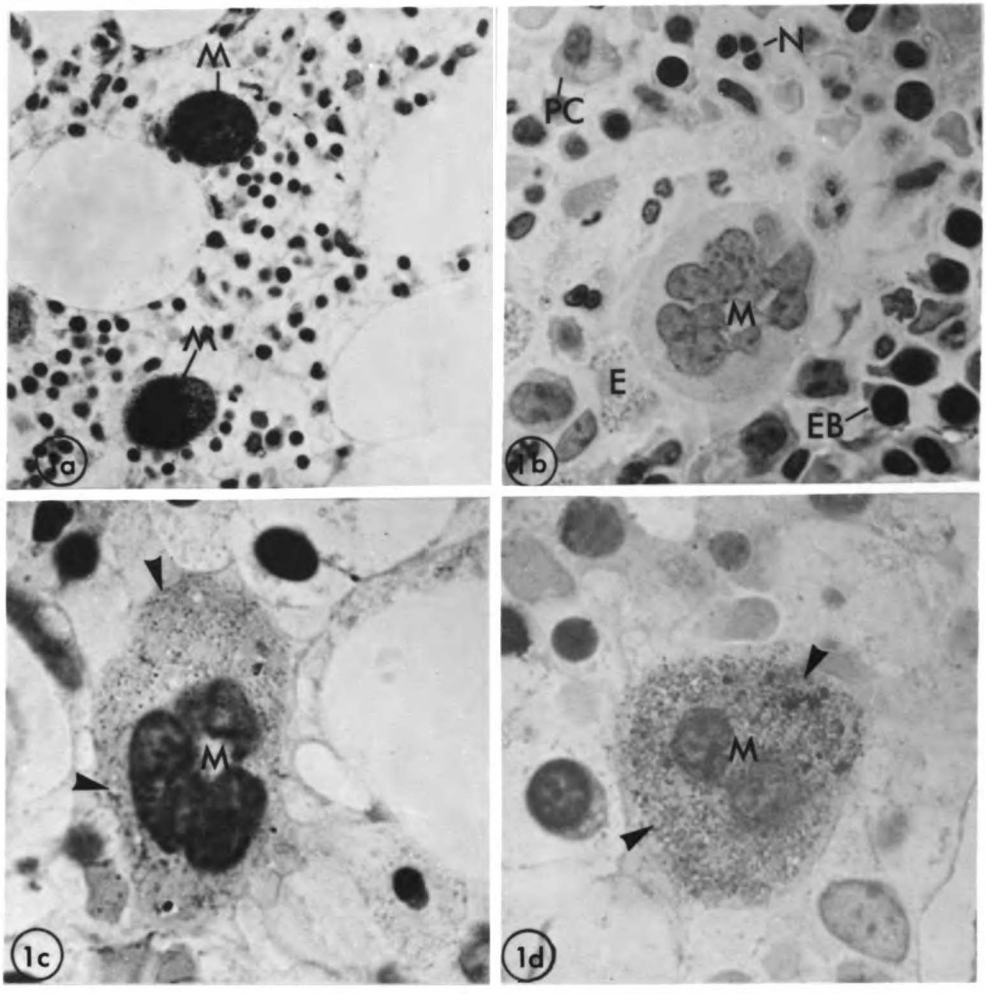
FIGURE LEGENDS

Figures 1 a - d. Light micrographs of 2 μm sections of plastic-embedded normal human bone marrow, processed for the immunocytochemical demonstration of platelet-specific proteins. In a and c, the sections were exposed to anti-βTG antiserum (prepared in our laboratory). In figure b the section was exposed to normal rabbit serum, and in figure d the section was exposed to anti-PF4 antiserum. All sections were then exposed to protein A-peroxidase conjugate and treated with diaminobenzidine and hydrogen peroxide. a. Two megakaryocytes (M) show dark intracytoplasmic staining while other cells are unreactive. X 600. b. Control preparation. A megakaryocyte (M) in the center of the field, easily distinguished by its size and nuclear morphology, shows no peroxidase label. The other cells were also unreactive. N, neutrophil; PC, plasma cell; EB, erythroblasts. Eosinophil (E) granules can be distinguished by their size and optical properties, but are not reactive. X 1,300. (c) A megakaryocyte (M) shows a granular pattern (arrows) of intracytoplasmic peroxidase label, similar to the known distribution of alpha granules in the megakaryocyte (22, 23). The other cells are unreactive. X 1,900. (d) A megakaryocyte (M) in the center of the field shows a striking granular pattern (arrows) of intracytoplasmic label. The other cells are unreactive. X 1,900.

Figure 2. Transmission electron micrographs of portions of human megakaryocytes processed with saponin for the immunocytochemical demonstration of βTG and PF4. (a) Control sample. Tissue was exposed to normal rabbit serum followed by Fab-peroxidase. No reaction product is seen

anywhere in this cell. X 32,000. (b-d) Tissue exposed to anti-βTG serum followed by Fab-peroxidase. (b,c) Peroxidase reaction product is seen in some Golgi cisternae (large arrows) and in a few vesicles (small arrows). (b) X 35,000; (c) X 56,000. (d) Two granules are reactive for βTG. X 56,000. (e) Tissue exposed to anti-PF4 serum, followed by Fab-peroxidase. A reactive granule is seen in this field. X 56,000.

All tissue was fixed in 2% paraformaldehyde for 2 hours and permeabilized with 0.02 - 0.1% saponin. (α), alpha granule. (DMS), demarcation membrane system. (m), mitochondrion; (g), granule.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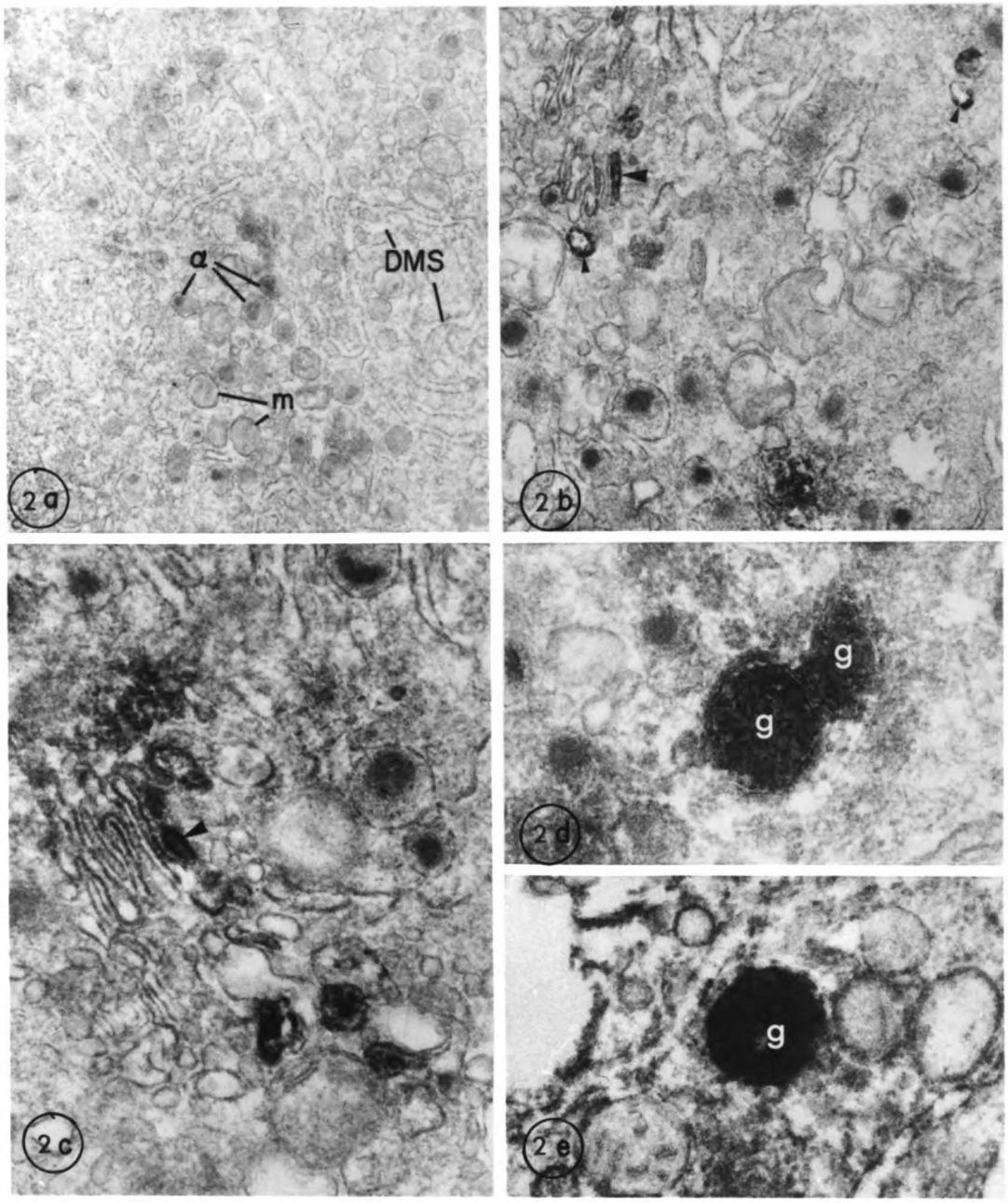
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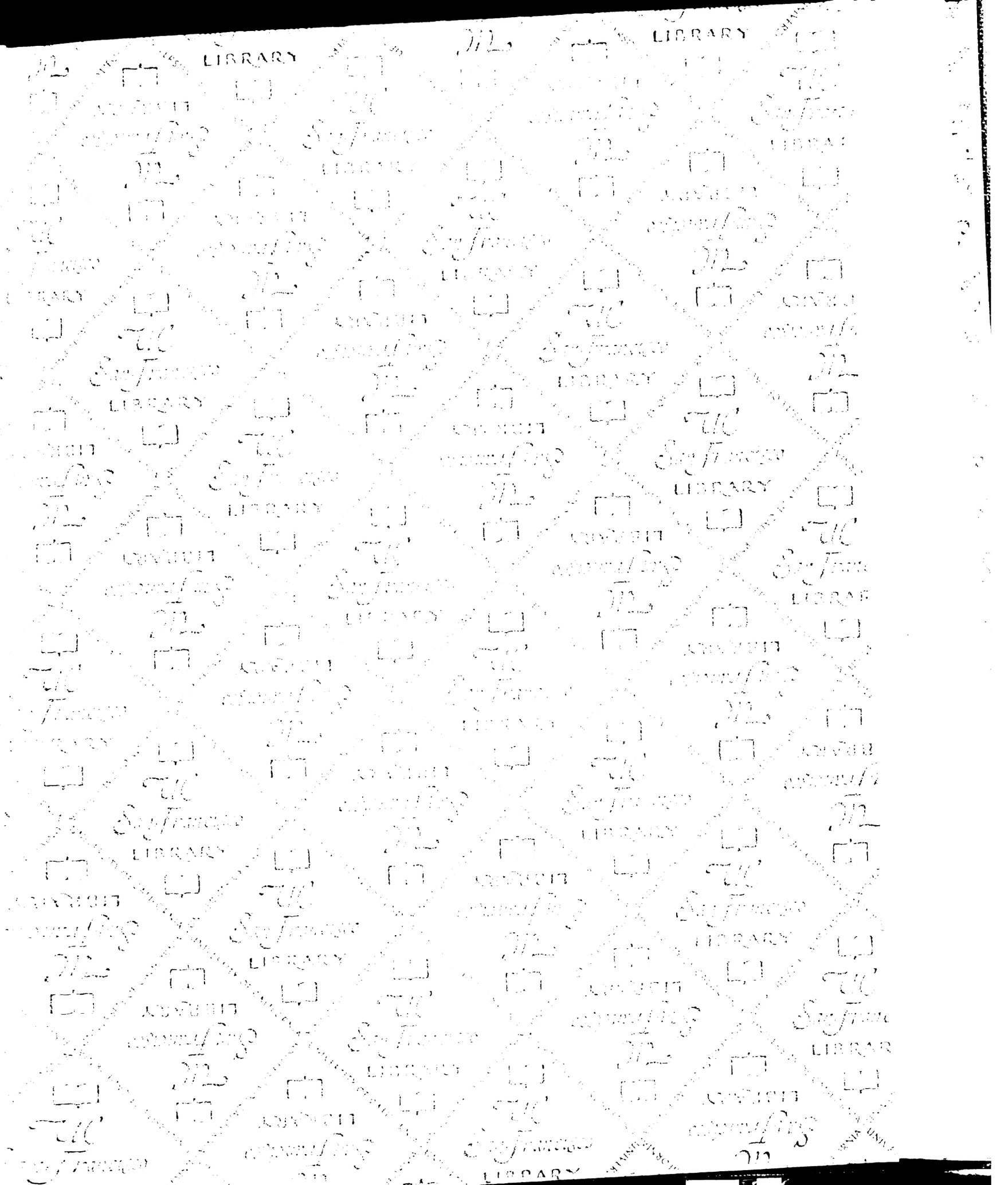
ADDENDUM

In order to fulfill university requirements for the format of the doctoral dissertation, all prints have been reduced in size from the original by approximately 30% (except Fig. 1 in Chapter 4, which is reduced in size by approximately 40%). The final magnifications quoted in this manuscript should therefore be adjusted accordingly.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