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USE OF SCANNING ELECTRON MICROSCOPY TO STUDY STRUCTURAL-FUNCTIONAL RELATIONSHIPS IN NORMAL AND DISEASED PLATELETS

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Abstract

This paper reviews the contribution of scanning electron microscopy (SEM) to our understanding of platelet physiology and pathology. Observations of platelet shape changes which accompany activation and the ability to visualize and analyze platelet aggregation and adhesion in three dimensions make this experimental medium an important tool in the evaluation of healthy and diseased platelets. While SEM adds a valuable third dimension to the study of morphology and ultrastructure, its greatest contribution is realized when studies are correlated directly with light and/or transmission electron microscopic observations and with studies of functional capacity.

KEY WORDS: Platelet ultrastructure, aggregation, adhesion, structural-functional correlations, scanning electron microscopy, transmission electron microscopy

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Introduction

Platelets are capable of activities as diverse as those seen in nucleated cells. They adhere to surfaces (Hellem 1960, Michaeli and Orloff 1976), undergo alterations in shape (Born 1970, White 1974b, Frojmovic and Milton 1982), secrete granule contents (White 1970,1973), interact with other platelets to form aggregates (Born and Cross 1963, Vargaftig et al. 1981), migrate directionally in response to chemotactic signals (Lowenhaupt 1982), engulf particles (Movat et al. 1965, Mustard and Packham 1968, White 1972a), are capable of internal contraction (White 1974a), and express procoagulant activity (Walsh 1978).

In the maintenance of hemostasis, adhesion, granule release ("the release reaction"), aggregation, and contraction are considered the basic platelet reactions (White 1982). While they usually occur together, any one of these functions can be expressed independently of the others.

Adhesion refers to the interaction of platelets with surfaces. Subendothelial structures including collagen and microfibrils provide the adherent surfaces during hemostasis (Baumgartner and Haudenschild 1972). In contrast, aggregation refers to platelet-platelet interactions. The combined effect of adhesion to subendothelial structures and platelet-platelet aggregation is required for the formation of a competent primary hemostatic plug (Baumgartner and Muggli 1976). Stimulation of the platelet membrane by platelet agonists results in mobilization of arachidonic acid for prostaglandin synthesis. Thromboxane A_2 (TxA_2) and cyclic endoperoxide intermediates are powerful aggregators and stimuli for granule release. They may act either indirectly via cyclic AMP (Salzman 1977, Smith 1981) or directly as ionophores to mediate calcium mobilization (Gerrard and White 1978). Calcium mobilization and prostaglandin (TxA_2) synthesis support cytoskeletal mediated internal contraction which is responsible for central migration of granules and microtubules following platelet stimulation and which contributes to the shape change associated with stimulation (Gerrard and White 1978). In addition, contraction potentiates granule secretion and provides the forces for clot

retraction, a process that stabilizes the fibrin-platelet network that forms during clotting. Granule secretion enhances platelet plug formation by release of ADP and serotonin, two powerful platelet agonists, from dense bodies into the immediate environment of the evolving aggregate, thus promoting its propagation (Holmsen et al. 1969).

Functional Anatomy

Interpretation of the three-dimensional data provided by SEM requires an understanding of platelet ultrastructure and function. White has introduced the concept of structural physiology to emphasize the close correlation of morphologic changes with physiologic and biochemical events in stimulated platelets (White 1971a). He has proposed subdivision of platelet anatomy into four functionally distinct zones: 1) the peripheral zone, 2) the sol-gel zone, 3) the organelle zone and 4) the membrane systems (White 1982).

The peripheral zone:

The peripheral zone is responsible for the platelet's ability to recognize and respond to a variety of stimuli. It bears the receptor sites for platelet agonists and inhibitors (Berndt and Phillips 1981b), and its unique composition is responsible for the platelet's ability to adhere to other platelets and to foreign surfaces (Berndt and Phillips 1981b, Nurden et al. 1981, Phillips 1980, Phillips et al. 1980, Nurden and Caen 1978). In addition, platelet support for plasma clotting is supplied by this zone which provides surface phospholipids (platelet factor 3 or PF 3) that catalyze several steps in coagulation (Fanti and Ward 1955, Walsh 1978).

The peripheral zone is composed of three morphologic domains: (1) the exterior coat or glycocalyx, (2) the unit membrane and (3) the submembranous zone. The peripheral zone extends into and lines the tortuous channels of the surface-connected open canalicular system (OCS) (Behnke 1967, 1970b) (Figure 1). This system of surface-connected channels gives the platelet a marked increase in surface area which Morgenstern and Stark (1975) suggest, based on morphometric analysis of the OCS in resting and stimulated platelets, may supply the membrane required for pseudopodia formation following platelet stimulation. Milton and Frojmovic (1979) came to similar conclusions from measurements of surface area differences in resting and stimulated platelets from normal and Bernard Soulier donors. In contrast, White and Clawson (1980) have demonstrated by freeze fracture and electron cytochemistry that channels of the OCS are seldom single channels, but rather are an anastamosing labyrinth of fenestrated channels that could not be easily evaginated. These authors conclude that the OCS could not provide membrane for pseudopod formation. Egress of granule contents through the OCS has been demonstrated (White 1973) and engulfment of particulates occurs via this system (Movat et al. 1965, White 1972a, Barnhart and Noonan 1978, White and Clawson 1981, 1982). While large particle uptake does not appear to represent phagocytosis (White 1972a), small particle uptake is associated with formation of phagolysosomes and thus would appear to be true phagocytosis (Lewis et al. 1976, White and Clawson 1982).

The exterior coat or glycocalyx is formed by the exterior projection of membrane glycoproteins and glycolipids. By negative stain whole mount electron microscopy it has been defined as a dense layer of 15-20 nm exterior to the trilaminar plasma membrane (Behnke 1967, 1968). Using cationic dyes, White (1971b) demonstrated that the platelet exterior coat is thicker and denser than that present in other blood cells. Despite its external orientation, the exterior coat is not appreciated by conventional SEM. Polyacrylamide gel electrophoresis of isolated platelet membranes demonstrates a pattern of membrane glycoproteins distinct from those of other blood cells (Phillips and Agin 1977, Nurden et al. 1981, George 1978). Of the approximately 50 polypeptides identified in isolated platelet membranes (Sixma et al. 1982b) eight glycoproteins (GP Ia, Ic, IIa, Is/Ib, Id, IIb, III, IV) are externally oriented and can be readily labelled by non-penetrating radioactive probes (Phillips 1980, Nurden et al. 1981, Peterson 1982). More recently an additional glycoprotein, GP V, has been shown to be externally oriented (Berndt and Phillips 1981a). The external orientation of these glycoproteins contributes to the carbohydrate rich glycocalyx which gives the platelet membrane its unique properties. Three of these (GP Ib, IIb, and IIIa) have been implicated in platelet adhesion-aggregation reactions and will be discussed in further detail under specific disorders of platelet function.

The platelet unit membrane is a trilaminar structure which demonstrates no differences from other biologic membranes by transmission electron microscopy (Figure 1). However, replicas of freeze fractured platelets reveal differences in the number and distribution of intercalated particles on the exoplasmic (E) and protoplasmic (P) face of platelet membranes, a finding consistent with the differences in transmembrane protein composition of these two membrane leaflets (White and Conard 1973). The platelet membrane contains various enzyme systems including $Na^{+}K^{+}$ and $Ca^{++}Mg^{++}$ ATPases (Chambers et al. 1967, White and Gerrard 1980) and adenylate cyclase (Haslam 1973, Mills and Macfarlane 1976).

The phospholipid orientation in the platelet membrane is asymmetrical (Schick et al. 1976). During platelet stimulation reorientation of membrane lipids may provide exposure of those phospholipids which confer platelet coagulant (PF 3) activity (Fanti and Ward 1955).

The submembranous zone is defined as that membrane associated area which lies just internal to the plasma membrane but peripheral to the microtubular coil (White 1982). Its composition is not well defined but the area is thought to contain a regular system of filamentous elements (White 1969), best visualized in osmotically shocked platelets (Zucker-Franklin 1970). This zone of dense filaments appears to exclude

organelles from the submembrane region. The participation of the submembranous cytoskeleton in the formation of membrane-cytoskeleton linkages seems likely. Debus et al. (1981) have demonstrated concentration of two actin modulating proteins, actin binding protein and alpha-actinin in this region as well as within pseudopods. Phillips et al. (1980) have isolated tritonized cytoskeletons from thrombin stimulated aggregates and by SDS gel electrophoresis have demonstrated the presence of 2 membrane glycoproteins (GP IIb-IIIa) associated with cytoskeletons enriched in actin, actin binding protein, myosin, a 90K Dalton and a 56K Dalton protein. As compared to cytoskeletons from non-aggregated thrombin-stimulated platelets in which membrane components were no longer present, there was a significantly greater amount of actin binding protein present when membrane glycoproteins were linked to cytoskeletons. In addition the 90K and 56K components were not identified in nonaggregated platelets. Schollmeyer et al. (1978) demonstrated in vitro that platelet actin binding protein promotes actin filament crosslinking and that alpha-actinin will produce lateral association of cross-linked actin filaments into parallel bundles. The presence of accessory proteins which promote the association of actin into parallel filament bundles suggests that this submembranous region may be involved in pseudopod formation (White 1982).

Sol-Gel Zone:

Previously referred to as the hyaloplasm, the sol-gel zone is the viscous cell matrix which contains proteins capable of being assembled into various filament systems that make up the platelet cytoskeleton. This complex system of microfilaments, microtubules, and accessory/regulatory proteins controls cell shape and provides the motile forces for internal contraction and granule secretion. Platelet microtubules are involved in maintenance of the discoid shape of the resting platelet (White 1968a, White and Krivit 1967). In sectioned platelets they measure 25 nm in cross-sectional diameter (White 1971a) forming a circumferential bundle of 8-20 microtubules lying just under the platelet membrane (Behnke 1970c) (Figure 1). Nachmias et al. (1980) have presented evidence that the peripheral bundle is composed of a single coiled microtubule.

An impressive group of proteins associated with the cytoskeleton have thus far been identified biochemically in platelets. These include actin (Probst and Luscher 1972), myosin (Booyse et al. 1971, Adelstein et al. 1971, Pollard et al. 1974), tropomyosin (Cohen and Cohen 1972), tubulin (Crawford and Castle 1976) actin binding protein (Lucas et al. 1976, Rosenberg et al. 1981), alpha-actinin (Rosenberg and Stracher 1981, Puszkin et al. 1978), vinculin (Billet et al. 1981) and P235 (Collier and Wang 1982) as well as several regulators of actin polymerization (Harris and Gooch 1981, Markey et al. 1981, Wang and Bryan 1981, Lind et al. 1982). The platelet contains actin in excess of myosin in a molar ratio of 100 to 1 (Pollard 1975). This suggests that actin has a role in platelet structure independent of its role in force generation via actomyosin. Lind and Stossel (1982) review the evidence that pseudopod formation is controlled by rapid actin polymerization but is not dependent on myosin. In addition, the presence in platelets of a microtrabecular filament network similar to that described in other cells (Buckley and Porter 1975, Wolosewick and Porter 1979, Buckley and Raju 1976) has been suggested by stereo whole mount electron microscopy (Lewis et al. 1980) and by transmission electron microscopy (TEM) of detergent treated whole mounts of adherent spread platelets (Mattson and Zuiches 1981b). Several recent reviews on the role of the platelet cytoskeleton in platelet physiology are highly recommended (Adelstein and Pollard 1978, Cohen et al. 1979, Gerrard et al. 1981, Lind and Stossel 1982, Fox and Phillips 1983, Nachmias 1983).

Organelle zone:

Organelles within this zone include mitochondria, glycogen particles, peroxisomes, dense-core granules, alpha granules, and lysosomes (Figure 1). In response to a number of stimuli platelets secrete the contents of their dense-core granules and alpha-granules. This secretory response is known as the "release reaction" (Grette 1962). Strong stimuli such as thrombin and high concentrations of collagen may also induce secretion of acid hydrolases from lysosomes (Kaplan 1981). The granules play an important role in the functional capacity of the platelet. The dense-core granules contain non-metabolic adenine nucleotides (ADP and ATP), calcium and serotonin. Release of ADP promotes second wave aggregation in vitro. Inhibition of granule release by aspirin and other nonsteroidal anti-inflammatory drugs is accompanied by a measurable loss of second wave aggregation in vitro and a variable prolongation of the bleeding time.

Alpha granules contain platelet factor 4 (PF4), beta thromboglobulin (BTG), platelet fibrinogen, von Willebrand factor (factor VIII:VWF), fibronectin, and platelet derived growth factor. The contents of platelet granules are released by means of exocytosis (Holmsen 1965) into the surface connected canalicular system (White 1973).

Further information on platelet granule biochemistry, cell biology, morphology and pathology is provided by several recent reviews (Kaplan 1981, Holmsen 1980).

The membrane systems:

Channels of the surface-connected open canalicular system (OCS) and the dense tubular system (DTS) make up the major part of this compartment. These two membrane systems are often found in intimate association with one another (Breton-Gorius and Guichard 1972, White 1972b). The DTS, distinguished by its content of amorphous material (Behnke 1970b), is the site of calcium sequestration in the platelet (Gerrard et al. 1978); it has been likened to the sarco-

tubules of muscle (White 1975) while the OCS has been compared to the transverse tubules of muscle. Ca^{++}/Mg^{++} ATPase activity is localized in the membranes of the DTS (Cutler et al. 1978) and calcium sequestration by the DTS is promoted by rises in platelet cyclic AMP. Many platelet inhibitors act by stimulating platelet adenylate cyclase to cause a rise in cyclic AMP (Haslam 1973, Haslam et al. 1977). There is evidence that the DTS with its peroxidase activity is the site of prostaglandin synthesis (White 1972b, Gerrard and White 1978, Breton-Gorius and Guichard 1972). Platelet stimulation results in release of arachidonic acid from phospholipids in the platelet membrane. Arachidonic acid in turn is metabolized to yield thromboxane A_2 (TxA_2), a potent vasoconstrictor and aggregator of platelets (Hamberg et al. 1975). Two important enzymes in TxA_2 synthesis are cyclooxygenase and thromboxane synthetase. Production of TxA_2 can be blocked by acetylation of cyclooxygenase by aspirin. Inhibition of TxA_2 synthesis results in inhibition of ADP release and second wave aggregation. Prostaglandin synthesis in endothelial cells results in production of prostacyclin (PGI_2), a powerful disaggregator and inhibitor of platelet aggregate formation as well as a vasodilator (Moncada et al. 1976). Alternate pathways for platelet activation may involve release of lysophosphatidic acid from membrane phosphotidylinositol as this component is an effective platelet aggregator (Beauton et al. 1982).

It is clear that an intact endothelial surface, with its antiaggregatory PGI_2 activity, is a powerful deterrent to inappropriate thrombus formation. Loss of endothelial cells results in localized loss of this inhibitory activity and allows accumulation of platelets.

The interaction of PGI_2 and TxA_2 as modulators of platelet function appears to be through control of calcium flow from the DTS. Evidence supports PGI_2 action via a rise in cyclic AMP which in turn activates a protein kinase that phosphorylates a protein regulator of calcium uptake into the DTS (Gerrard et al. 1978). Active prostaglandins (PGG_2, PGH_2, TxA_2) do not measurably lower cyclic AMP, thus their action may be directly on calcium release, acting as ionophores. For a detailed discussion of the evidence in support of such a mechanism, the reader is referred to the review by Gerrard and White (1978). Additional reviews of the role of prostaglandins in platelet function are provided by Nalbandian and Henry (1978) and Cohen (1980).

Platelet Shape

Platelets are exquisitely sensitive cells which respond to minor mechanical and chemical stimuli by alterations in form and functional state. This sensitivity makes it very difficult to be certain that platelets under study are in fact "resting" and not altered by some stimulus. For this reason the search for appropriate anticoagulants and separation procedures, which preserve the platelet in a form that closely approximates its native state, has been extensive. Scanning electron microscopy has played a major role in revealing the precise form of the unactivated platelet and has also contributed to our understanding of the complex process of "shape change" that accompanies platelet activation (i.e. aggregation and adhesion reactions).

The resting platelet (Figure 1A-C):

Preservation of platelets in their unstimulated, circulating state is best achieved by drawing whole blood directly into aldehyde fixative (Hovig 1970a, Barnhart and Riddle 1967, Barnhart et al. 1972, Hattori 1972). By this technique the majority of platelets will appear as biconvex discs with no, or only a few, small pseudopods (Figure 1A). Quantitation of the number of pseudopods in directly fixed platelets has been performed by several authors with good agreement. Barnhart and associates have found that 65-70% of directly fixed platelets are smooth and free of pseudopods in both dogs (Barnhart and Riddle 1967) and humans (Barnhart et al. 1972). The remaining platelets showed minor signs of activation; most of these remained flat discs with 1 or 2 small thin projections. By SEM, Hattori et al. (1969) found pseudopods to emerge exclusively from the marginal portion of the platelet at early stages of activation. Bifurcation of pseudopods has been described (Larrimer et al. 1970). Diameters of discoid platelets range from 2-4 µm with an average thickness of 0.4 to 0.8 µm (Hovig 1970a). Bigel and associates (1967) found the average diameter for normal adults to be 3.1 µm. The surface of the unstimulated platelet appears rough in comparison to red cells, and occasional small protuberances and several pit-like openings can be seen on the platelet surface (Figure 1A). While as many as 30 or more surface openings may be seen by freeze fracture or thin section TEM (Werner and Morgenstern 1980), they are often difficult to visualize by SEM presumably due to their small size. By comparing SEM with TEM of the resting platelet, these surface pits correspond to openings of the surface-connected open canalicular system (Figure 1B). Werner and Morgenstern (1980) found surface openings more concentrated near the periphery of the discoid platelet, but we have not seen this preferential distribution.

TEM of the resting platelet demonstrates random dispersion of organelles such as granules, mitochondria and glycogen particles. Microfilaments are not discernible by ordinary TEM techniques. Microtubules, on the other hand, are readily seen in sectioned platelets as a marginal band consisting of 8-20 individual microtubules (Behnke 1970a, 1970c, White 1971a, Zucker-Franklin 1969) (Figures 1B,C). Nachmias et al. (1977, 1980) have been able, after detergent extraction, to follow the peripheral microtubule for several turns and conclude that the microtubule bundle is composed of a single long microtubule arranged in a coil. Free ends never exceeded two, lending more evidence to Behnke's (1970c) earlier suggestion that the peripheral microtubule bundle is composed of a single long coiled microtubule.

This coil of microtubules maintains the

discoid shape of the resting platelet. Evidence for this was obtained from experiments which showed that agents causing depolymerization of microtubules, i.e., cold (White and Krivit 1967, Zucker and Borelli 1954, Hovig 1970b) and colchicine, vincristine and vinblastin (White 1968a, Hovig 1970b) result in disc to sphere transformation of platelets.

Effects of preparative procedures on platelet structure and function:

Most studies of platelet function require collection of platelets in a solution appropriate for experimentation. Thus anticoagulated suspensions of platelets in native plasma or an appropriate buffer are necessary. Clearly the mechanical stress associated with sample collection and centrifugation as well as the effects of various anticoagulants and salts can all influence the structure and function of the final product.

The size of the needle used for venipuncture has been shown to have an effect on platelet structure. Hattori et al. (1977) found that a 23 gauge needle produced 50% fewer disc-shaped (i.e. unstimulated) platelets than blood drawn through an 18 gauge needle.

The choice of anticoagulant is especially important. Platelets drawn into citrate prior to fixation will most closely resemble directly-fixed platelets; nevertheless they often show variable numbers of atypical forms including spherical, elongated, and ellipsoid forms (Hattori et al. 1969, Zucker and Borelli 1954) (Figure 2A-C). Larrimer et al. (1970) demonstrated that ACD, modified ACD and heparin anticoagulation produce platelets (in PRP) similar in morphology to those in citrate. Zucker and Borelli (1954) demonstrated that temperature plays a major role in preservation of resting morphology; platelets collected in citrate and oxalate and maintained at 37°C were mainly discoid (85%) even though two thirds of the discoid platelets contained small pseudopods. At 25°C fewer platelets remained discoid. Nachmias (1980) and Hovig (1970a,b) have confirmed the beneficial effect of processing at 37°C.

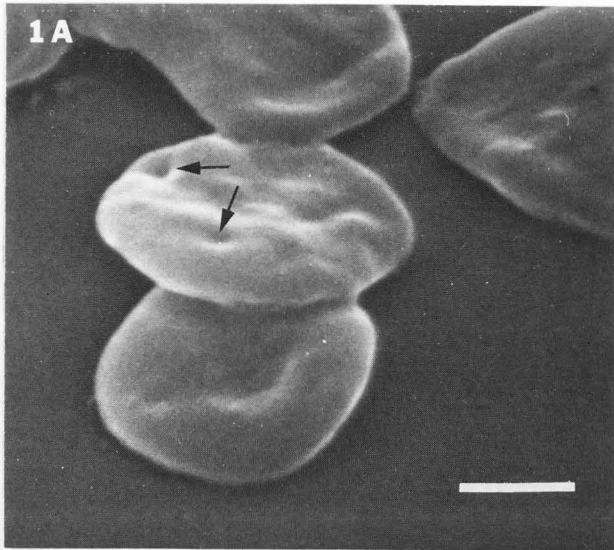
Phase microscopy shows that platelets exposed to cold (0-4°C) undergo shape changes converting from disc to "spiney sphere" (Zucker and Borelli 1954). By SEM these spiney spheres have highly irregular surfaces with numerous bud-like pseudopods (Hovig 1970a,b) and only infrequent thin elongated pseudopods (Larrimer et al. 1970). Sphering is the result of cold-induced depolymerization of the marginal band of microtubules (White and Krivit 1967). No evidence of internal contraction, granule centralization or central migration of microtubules has been described by TEM. Rewarming to 37°C results in recovery of the discoid shape in about 30% of platelets, associated with reformation of the microtubule bundle (White and Krivit 1967).

Colchicine, which also depolymerizes microtubules, causes platelet sphering (White 1968a, Behnke 1970a) in vitro as well as in vivo (Hovig 1970b). As with cold treatment depolymerization of microtubules and sphering with pseudopod formation are the major findings by TEM (White 1968a). However colchicine induces changes in the DTS and produces inclusions which are not described with cold (White 1968b).

While EDTA may be the preferred anticoagulant for certain preparative techniques, it should be kept in mind that it is a poor choice for preservation of resting morphology. Platelets exposed to EDTA at 37°C lose their disc shape and become irregularly swollen with multiple pseudopods by phase contrast microscopy (Zucker and Borelli 1954) (Figure 3A,B). Externally they appear similar to platelets sphered by exposure to cold, treated with colchicine or stimulated by ADP and thrombin. TEM studies (White 1968c), however, have demonstrated internal changes that are quite distinct from those present following stimulation with aggregating agents. The most prominent change is dilatation of the OCS. In contrast to cold and colchicine treatment, microtubules remain intact in their usual submembranous position. Furthermore, granules do not migrate centrally as they do following ADP or thrombin stimulation. In White's study (1968c) these alterations were present as early as 13 min after exposure to EDTA. Prolonged incubation in EDTA (greater than 3-4 hours) leads to granule dissolution and swelling with an apparent increase in mean platelet volume by Coulter counter electronic measurement (Mundschenk et al. 1976).

If experimental procedures require separation of platelets from plasma, the maintenance of normal morphology and function is more difficult. In general, platelets separated from plasma by a variety of techniques including differential centrifugation (Mustard et al. 1972), gel filtration (Tangen et al. 1973) and albumin density gradient centrifugation (Walsh 1972) show comparable functional capacities if care is taken to avoid low temperature, excessive mechanical stimulation, the introduction or release of agonists such as ADP, and depletion of plasma proteins required for function (Mason et al. 1974). Functional differences are slight as measured by aggregability, adhesion to glass, and content and release of adenine nucleotides (Mason et al. 1974). In contrast, morphology is not equally preserved by these techniques (Zucker et al. 1974).

Differential centrifugation by the Mustard technique (Mustard et al. 1972) at 37°C using ACD anticoagulant produced the best preserved resting morphology as studied by TEM. The important features of this technique are the use of 37°C rather than room temperature, the presence of apyrase to inhibit any nucleotide released during preparation, and the phosphate carbonate buffer which supports preservation of platelet shape and function. In this same study gel filtration at room temperature produced moderate irregularities in morphology with a few pseudopods. However platelets in general remained discoid and organelles remained dispersed. Gel filtration is preferred to centrifugation by some investigators (Akkerman et al. 1978). Platelets separated by gel filtration at 37°C are less activated than those separated at room temperature (Nachmias 1980). Of the three separation techniques, albumin density gradient centrifugation showed the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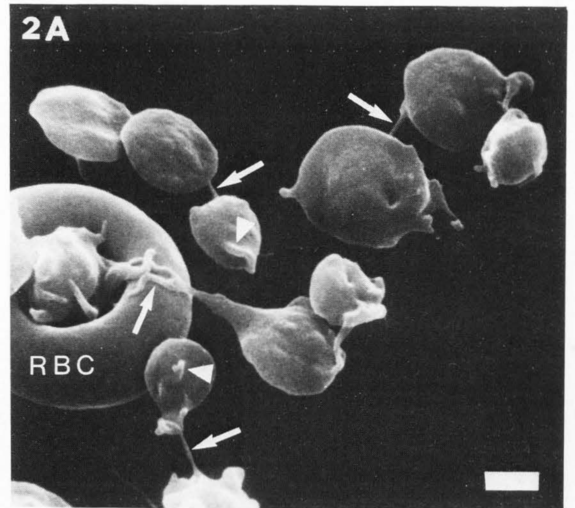
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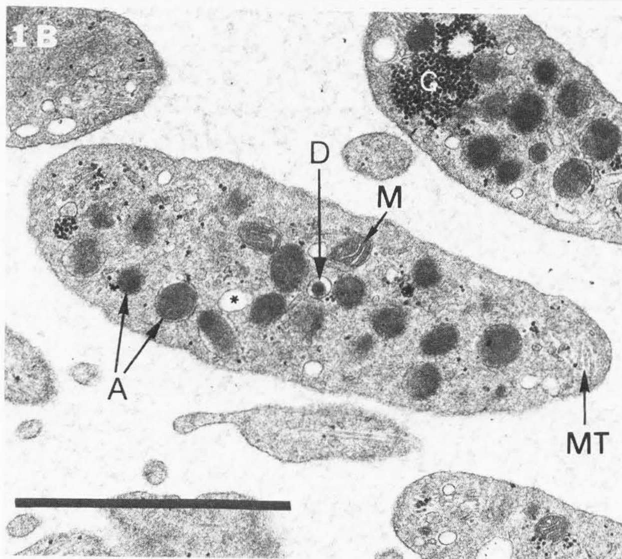
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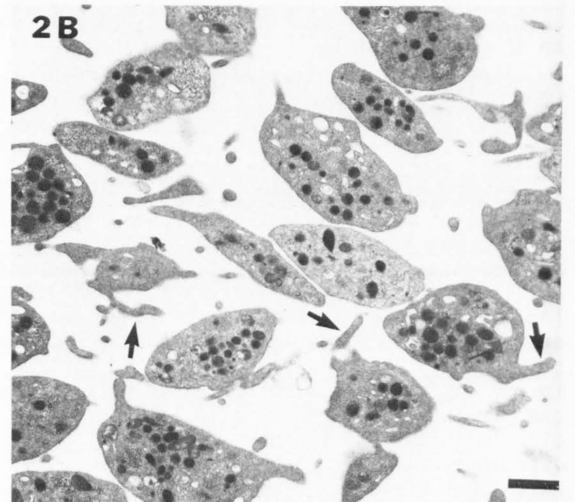
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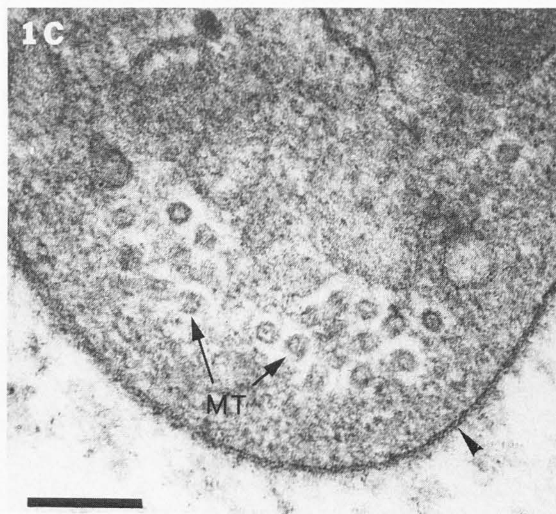
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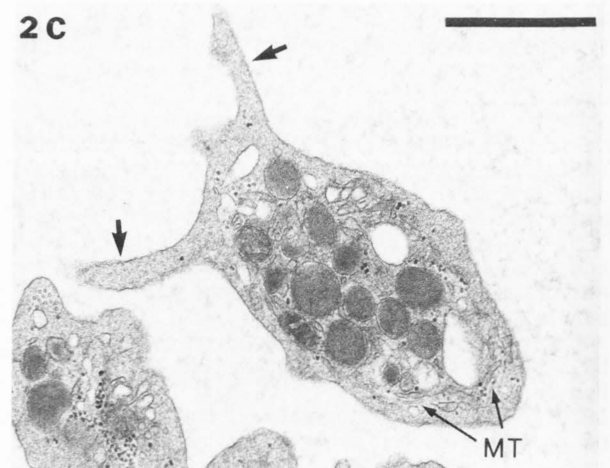
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MT

Figure 1 A-C. Ultrastructure of human discoid unstimulated platelets fixed immediately by drawing blood directly into glutaraldehyde. A: SEM. B and C: TEM. The discoid shape of the unstimulated platelet is best appreciated by SEM in A. Openings of the surface connected open canalicular system (OCS) are barely perceptible as minute pit-like depressions on the surface (arrows). Position of underlying granules is not discernible on the platelet surface. In the transmission electron micrograph B, discoid unstimulated platelets show randomly dispersed organelles including dense core granules (D), alpha-granules(A), mitochondria (M), and masses of glycogen particles (G) as well as channels of OCS (*). Dense core granules are distinguished from alpha-granules by their bull's eye appearance. The peripheral band of microtubules (MT) is seen in cross section in micrographs B and C at the poles of the discoid platelets. The trilaminar structure of the plasma membrane is evident in C (arrowhead). A and B: Bars = 1 micrometer; C: Bar = 0.1 micrometer.

Figure 2 A-C. Ultrastructure of platelets fixed after the preparation of citrated platelet-rich plasma (PRP). A: SEM. B and C: TEM. In the scanning micrograph A, note the mild activation that occurs during the preparation of PRP. Short blunt (arrowheads) and elongated thin (arrows) pseudopods are numerous. Most platelets remain discoid but occasional platelets are sphered. A red cell (RBC) is present in the preparation. Transmission electron micrographs of platelets fixed in PRP, B and C, show the preservation of the discoid shape as well as random disperson of organelles, and absence of a contractile wave. The main manifestation of early, mild stimulation is pseudopod formation (arrows). Higher magnification in C demonstrates preservation of the peripheral bundle of microtubules (MT). Thin pseudopods appear to preferentially form from the margin of discoid platelets (arrows). Bars = 1 micrometer.

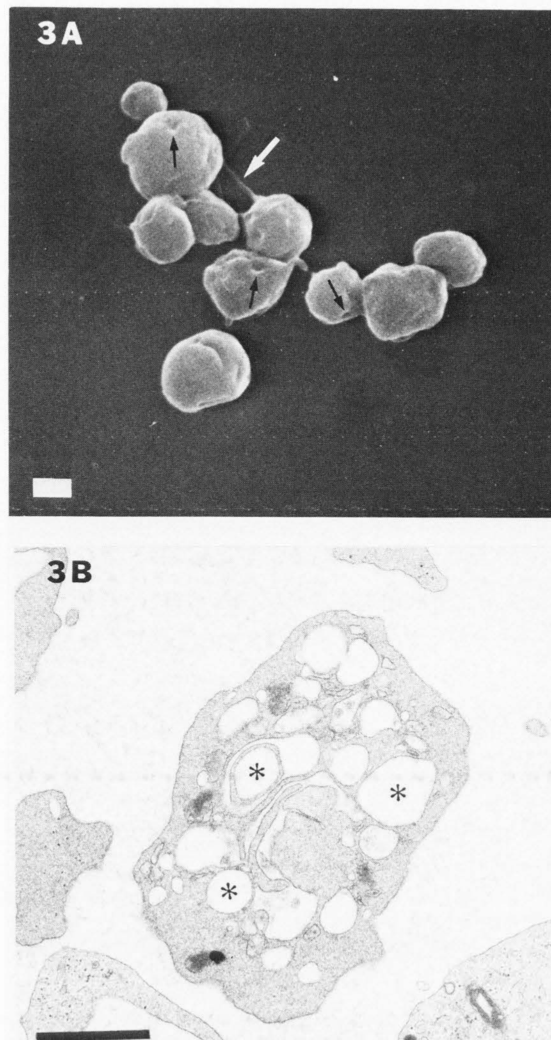


Figure 3 A-B. Ultrastructure of platelets collected in EDTA anticoagulant. A: SEM. B: TEM. Note loss of discoid shape. Sphered platelets show infrequent pseudopods (white arrow). Openings of the OCS are visible as pits on the surface (black arrows). Transmission electron micrograph of EDTA-anticoagulated platelets in B shows absence of microtubules, marked dilatation of OCS (*), and paucity of dense core and alpha-granules. Bars = 1 micrometer.

least preservation of morphology (Zucker et al. 1974). Improvements in albumin density gradient separation techniques which include addition of apyrase to the separation buffer are reported to have improved the preservation of ultrastructure with this procedure (Walsh et al. 1977). Among the newer separation techniques, Stractan gradients have been reported to preserve platelet structure while metrizamide produces structural alterations (Corash et al. 1977). Choice of buffer will also influence the final product. Tris buffers produce morphologic (Zucker et al. 1974) as well as functional abnormalities (Lages et al. 1975) while imidazole and triethanolamine buffers produce functional defects (Davis and Phillips 1971). Frojmovic and Milton (1982) further recommend that an atmosphere of 5-6% CO_2 and a pH of 7.4 $\pm$ 0.1 are optimal.

Glutaraldehyde in a wide range of concentrations (0.1 - 7.1%) is an effective fixative for both scanning and transmission electron microscopy of platelets (Barnhart and Riddle 1967, Larrimer et al. 1970, Hovig 1970a,b, Barnhart et al. 1972, Hattori 1972, Mattson et al. 1977). Large volumes of fixative-buffer solution containing relatively low concentrations of glutaraldehyde are recommended when platelets are to be fixed in whole blood or plasma in order to avoid coagulation of plasma proteins (Bessis and Weed 1972, White 1968d, Mattson et al. 1977). Buffer concentration appears to be more critical than glutaraldehyde concentration in preservation of ultrastructure as buffer is more freely diffusible than glutaraldehyde into the cell and therefore produces a greater osmotic effect (Arborgh et al. 1976, Barnhart and Riddle 1967). We have found that initial preservation in 0.1% glutaraldehyde in 0.1M cacodylate buffer pH 7.4 gives excellent preservation for both SEM and TEM

(Mattson et al. 1977). While many early studies of platelet three-dimensional structure yielded useful information from air dried preparations, for optimal preservation of orientation of pseudopods and avoidance of collapse of cells, critical point drying (Polliack et al. 1973) or lyophilization (MJ Murphy 1972) is preferred.

Shape Change

Morphologic changes have been observed in platelets both in response to stimuli which initiate aggregate formation and as part of the platelet response during adhesion to surfaces. While these morphologic changes closely parallel the biochemical and functional alterations which occur during aggregation, granule release, and adhesion, they appear to be separable events. The search for the role of shape change in these functional reactions has led to a variety of structural-functional studies employing numerous agonists and inhibitors. While these studies have clearly indicated that certain morphologic events can be correlated to structural rearrangements within the platelet, the role of shape change in augmenting platelet function has yet to be fully elucidated.

Morphologic alterations that accompany aggregation:

The platelet's response to stimuli is dependent on the nature, strength, and duration of the stimulus as well as the responsiveness of the platelet itself. With suboptimal stimuli, activation will be transient and reversible but with stronger stimuli, irreversible activation will occur (Zucker and Peterson 1970, Larrimer et al. 1970, Barnhart et al. 1972).

The addition of most platelet stimulators to platelets in suspension results in dramatic morphologic changes commonly referred to as platelet "shape change". Documentation of shape change may be based on direct microscopic observation or on indirect measurement of light transmission by aggregometry (Born 1970). With aggregometry, disc to sphere transformation is observed in a stirred suspension of platelets as a decrease in light transmission as well as a decrease in the amplitude of the recorder pen oscillations (Latimer et al. 1977, O'Brien 1970). The change in amplitude is due to light-deflecting differences between discs and spheres. Because discoid platelets are asymmetrical, they are oriented by the shearing forces of the stir bar to produce a visible swirl which is recorded as rapid oscillations. Immediately after addition of an aggregating agent, discoid platelets transform into spheres which no longer show the swirl effect and the oscillations disappear.

By SEM, the two major features of agonist-induced shape change are sphering and extension of surface projections (White 1974b). These surface projections are variably referred to as microspikes, filopodia (Nachmias et al. 1979) or pseudopods (Hovig 1970a,b, Barnhart et al. 1972). Surface projections may be short, blunt and generally rounded, or thin, elongated and cylindrical. When there is a preponderance of elongated, thin pseudopods, platelets are often referred to as "dendritic" in form (Bessis 1973, Hovig 1970b). For an excellent discussion of platelet shape change the reader is referred to the review by White (1974b).

ADP and thrombin (Figure 4A-G):

While platelets respond to many diverse stimuli, including mechanical shear forces, chemical agonists and inhibitors, and particulates such as latex, bacteria and collagen fibrils, the process of cell activation has been best worked out for ADP and thrombin stimulation.

Within seconds following activation by ADP (or thrombin), platelets transform from discs to spheres and numerous short bulbous and a few longer thin surface projections appear (White 1974b, 1968d, 1971b, Barnhart et al. 1972, Walsh and Barnhart 1973, Barnhart 1978, Larrimer et al. 1970, Shoop et al. 1970, Hovig 1970a,b, Hattori et al. 1969) (Figure 4A). Hattori (1972) suggests from observation of samples taken 5 seconds after ADP addition that bulbous, blunt projections arising from the flat face of the discoid platelet precede sphering. We have made similar observations (Mattson and Craft, unpublished observations) White (1974b) has observed that the earliest change is formation of short pseudopods most frequently at or near the edge of the disc. This type of pseudopod with its actin filament core is likely related to the activation-induced rapid polymerization of actin reported by many investigators, while the blunt knobby surface undulations seen in early stimulation may be related to the internal reorganization that accompanies internal contraction. We have never observed parallel bundles of actin filaments in these knobby, irregular surface convolutions (Figure 4B). While sphered platelets are often referred to as swollen, there is not general agreement that true volume increases accompany shape change. The mean platelet volume (MPV) of discoid platelets is approximately 4-8 μm^3 (Frojmovic and Milton 1982), while the reported MPV of EDTA sphered platelets varies from 7.7-10.3 μm^3 (Mundschenk et al. 1976). Variation in the reported volumes for sphered platelets appears to be due in part to differences in methodology and anticoagulant. In addition Bessman et al. (1982) have demonstrated that there is a nonlinear inverse relationship between MPV and platelet count which needs to be taken into account in interpretation of volume data. Investigators using Coulter counter measurements of mean platelet volume report increases in platelet volume (Bull and Zucker 1965, Laufer et al. 1979) while those estimating volume by microhematocrit (Born 1970) and microscopic measurements (Milton and Frojmovic, 1977, 1979) find no change in volume following agonist-induced shape change. For an in-depth discussion of surface area and volume change in stimulated platelets, the reader is referred to the recent review by Frojmovic and Milton (1982).

Following ADP induced shape change, loose aggregates initially form (Hovig 1970a, Barnhart et al. 1972) in which individual platelets retain their discrete identity (Figure 4C,D). Given a sufficient stimulus, these ultimately become tightly organized aggregates of fused platelets (Figure 4E,F). By SEM these irreversible aggregates resemble coral with numerous fingers and ridges formed by the fused platelet masses

(Figure 4E). With low concentrations of ADP irreversible aggregates do not form and disaggregation occurs; this is associated with restoration of the discoid form.

Changes in surface contour are associated with internal transformations. The randomly dispersed organelles of the resting platelet migrate centrally to form a nucleoid of granules and contractile filaments (Figure 4B). Gerrard et al. (1979) have demonstrated that the contractile wave is oriented toward the aggregate center and thus the centralized organelles and contractile apparatus will be displaced in this direction. This reorganization is associated with central migration of the peripheral microtubular bundle (White 1979) resulting in loss of the discoid shape and sphering. Central displacement of microtubules is apparently accomplished by depolymerization of tubulin with repolymerization at the new location. A transient decrease in polymerized tubulin content of platelets has been demonstrated immediately following stimulation by thrombin, ADP, epinephrine, or collagen with recovery of baseline polymerization by 1-4 min (Steiner and Ikeda 1979). While thrombin and ADP induced internal reorganization are identical, central fusion of granules has been reported with thrombin stimulation but not with ADP stimulation (White 1968b).

The state of microfilament polymerization in the resting and activated cell has also been an area of considerable interest since both cell shape and internal reorganization require participation of cytoskeletal components. In aggregation centralization of granules and microtubule coils is accompanied by a wave of contraction that is visualized as the appearance of a dense mass of poorly defined microfilaments in the cell center (White 1974a,b) (Figure 4B).

Because ultrastructural visualization of filament organization is exceedingly difficult, most studies on the state of contractile protein polymerization have used biochemical assays. Nachmias and her colleagues (1977, 1979), using the local anesthetics lidocaine and tetracaine which are inhibitors of microfilament polymerization, found that actin in the pseudopod-free, resting platelet is mainly monomeric or non-polymerized G-actin. A rapid increase in polymerized actin (F-actin) following chilling, thrombin and ADP stimulation has been demonstrated (Phillips et al. 1980, Jennings and Phillips 1981, Pribluda et al. 1981). Fox et al. (1981) found 40% of total actin was F-actin in resting platelets that were lysed under stabilizing conditions; this rose to 65% F-actin following thrombin stimulation. Others have reported between 20 and 50% F-actin in unstimulated platelets using a DNase I inhibition assay (Gitler et al. 1980, Carlsson et al. 1979, Jennings et al. 1981, Pribluda et al. 1981), even in the presence of tetracaine inhibition (Davies and Palek, 1982). F-actin in the resting platelet may represent a stable network of filaments responsible for the spatial relationships of organelles. This stable system of filaments presumably includes the submembranous filaments which are intimately associated with the microtubular coil on its internal side and the plasma membrane on its external aspect. Actin filaments which form following stimulation are present in bundles in pseudopods and within the platelet body (Nachmias 1980, Jennings et al. 1981, Gonella and Nachmias 1981). While actin bundles produced by stimulation are sensitive to depolymerization by cytochalasins, filamentous actin present in unstimulated platelets is not (Fox and Phillips 1981).

Agonist-induced cytoskeletal changes are associated with simultaneous membrane changes. Association of membrane GP IIb-IIIa complex with the underlying cytoskeleton occurs following thrombin (Phillips et al. 1980) and conconavalin A (Painter and Ginsberg 1982) aggregation but has not been observed following ADP aggregation (Rotman et al. 1982). These reported differences are surprising since ADP and thrombin produce almost indistinguishable morphologic changes (White 1968d) and both require fibrinogen binding to GP IIb-IIIa complex for aggregation to occur.

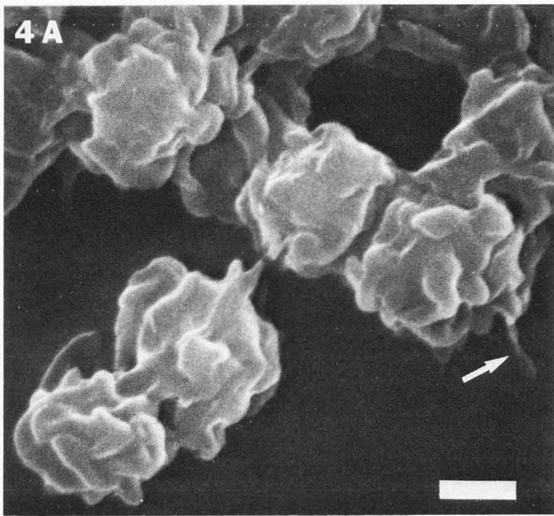
Epinephrine and other biologic amines (Figure 5A-C):

The question of whether shape change is required for either aggregation or granule release is raised by studies on epinephrine induced aggregation. This agonist produces a two-wave aggregation response similar to that seen with ADP. The primary wave is induced by the epinephrine while the second wave is mediated by thromboxane A_2 production and ADP release from dense-core granules. The primary wave of epinephrine aggregation, unlike ADP or thrombin stimulation, produces no shape change, no granule centralization and no contractile wave as evidenced by centralized filaments (Figure 5A-C). Nevertheless granule release can occur. A more recent role for epinephrine in platelet reactions has been postulated by Rao et al. (1980, 1981). These authors suggest that alpha-adrenergic receptor occupancy directly alters the platelet membrane, allowing irreversible aggregation to occur in response to a second agonist even in the absence of prostaglandin synthesis or release of granule constituents.

The effect of biologic amines on platelets is not uniform. Epinephrine, norepinephrine and serotonin induce aggregation and enhance spreading on a formvar surface (Barnhart et al. 1972). Serotonin, in contrast to epinephrine, produces platelet sphering with marked surface convolutions as seen by SEM. Another vasoactive amine, histamine, acts as a platelet inhibitor which diminishes contact-activated shape change (Barnhart et al. 1972).

Collagen:

Collagen is one of the strongest platelet agonists. Collagen induced shape change is similar in many respects to that seen with ADP and thrombin (Larrimer et al. 1970). Disc to sphere transformation is rapid and numerous long thin psuedopods are produced (Figure 6). Barnhart and Noonan (1978) demonstrated that as many as 16-20 pseudopods may be present per platelet. Terminal bulbous swellings have been described at the tips of pseudopods by SEM (Polasek 1982). By TEM these endings contain multivesicular structures (Warren and Vales 1972a, b).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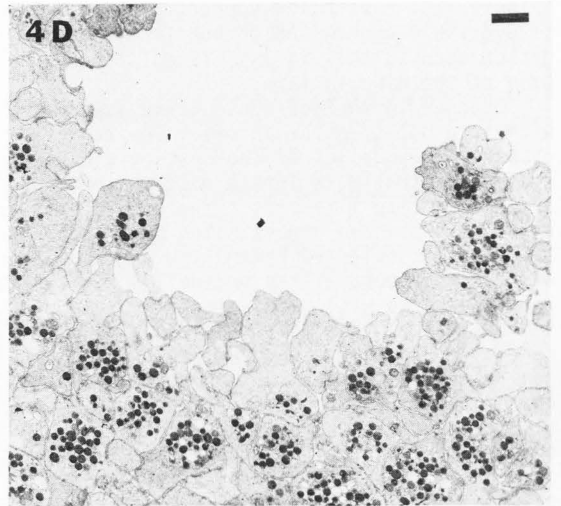
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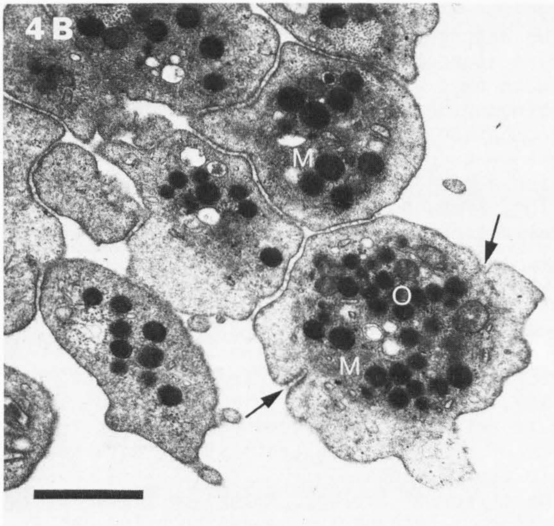
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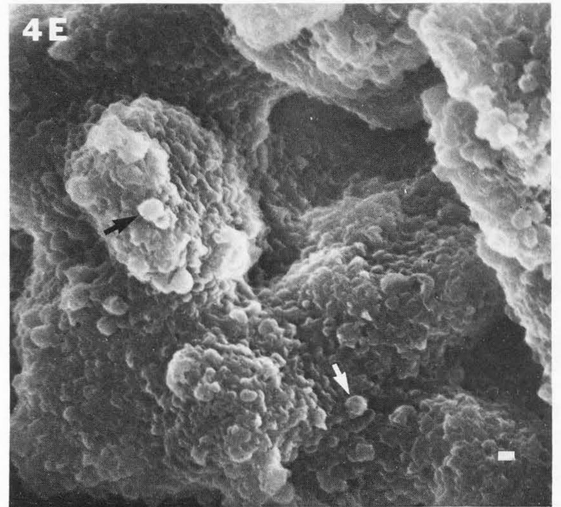
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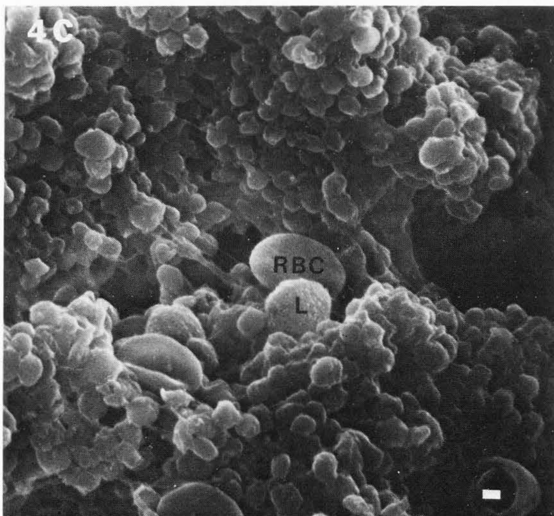
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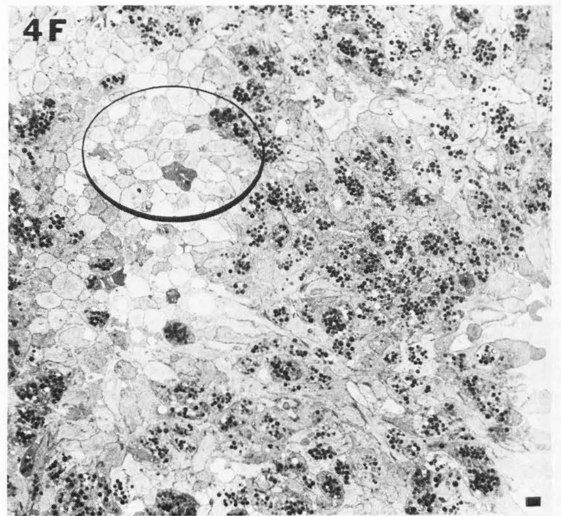
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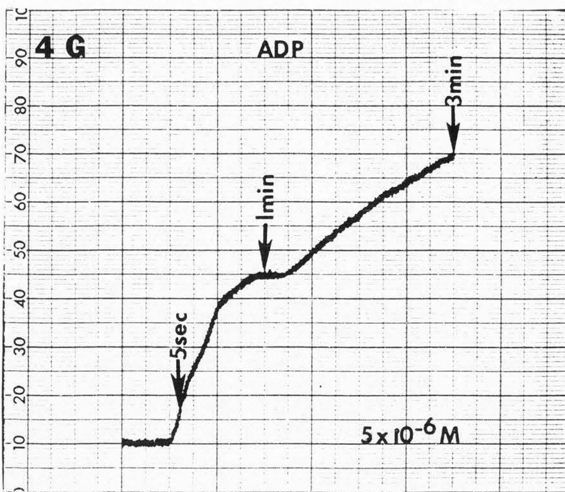


Figure 4 A-G. Ultrastructure of ADP aggregation. A and B: Samples harvested at 5 seconds after addition of ADP and examined by SEM (A) and TEM (B). C and D: Samples harvested at 1 minute and examined by SEM (C) and TEM (D). E and F: Samples harvested at 3 minutes and examined by SEM (E) and TEM (F). G: Typical 2 wave ADP aggregation curve demonstrating points of sampling. The earliest (5 second) shape change induced by ADP is seen in A and B. In A, SEM demonstrates the disc to sphere transformation. While thin pseudopods are present (arrow), the major surface alteration is the presence of blunt, short convolutions which give the platelets a cerebroid appearance. Openings of the OCS are not present on the crests of these convolutions as seen by SEM in A, but appear to exit from the bases of these projections as demonstrated by TEM in B (arrows). By TEM in B, centralization of organelles (O) is evident as is concentration of densely staining cytoplasmic matrix (M). This condensed matrix is presumably composed of contractile elements. Note the surface convolutions are devoid of organelles and the microtubule bundle is no longer present at the platelet periphery. C and D demonstrate a loose reversible platelet aggregate from a sample collected at the peak of primary wave aggregation (1 minute). By SEM (C), individual platelets are easily identified within the aggregate. Entrapped red cells (RBC) and a rare lymphocyte (L) are seen. TEM in D demonstrates that the integrity of individual platelets within the primary aggregate is preserved. In E and F the irreversible aggregates harvested at the peak of second wave aggregation (3 minutes) show loss of individual platelet integrity. By SEM (E), the aggregates resemble a coral reef with projection of wide ridges composed of fused platelets. The surface has a rough cobblestone appearance and distinct margins of platelets are not discerned. Occasionally an intact platelet is recognizable on the surface (arrow). By TEM (F), loss of platelet contents and focal disruption of membranes are seen (Circle). Bars =1 micrometer. (Micrograph A is reprinted with permission from Stain Technol 52: 151-158, 1977).

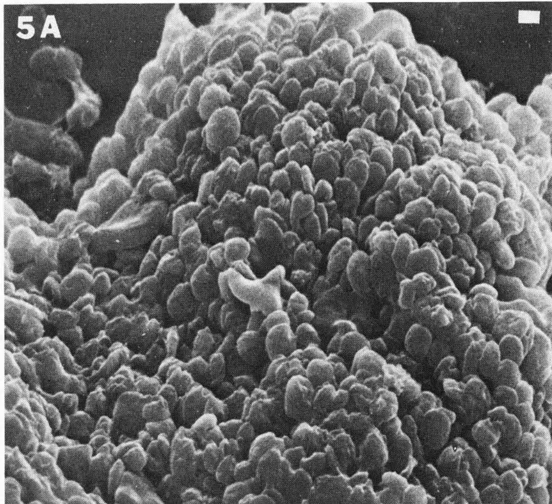
Platelets activated in vivo:

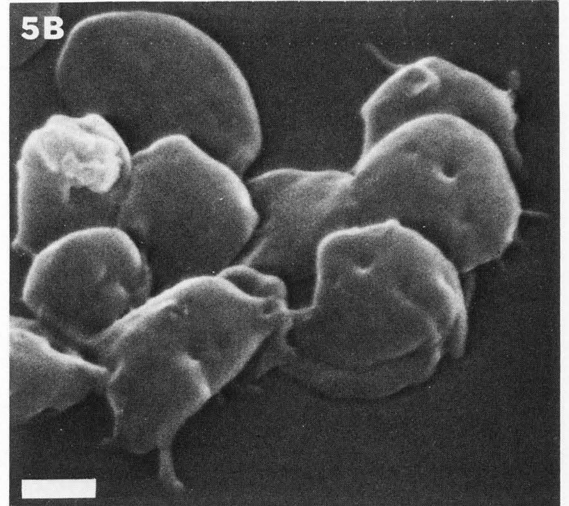
Increased numbers of abnormal forms and sphered platelets both with and without pseudopods have been identified by SEM in immediately fixed blood from patients with disseminated intravascular thrombosis (DIC), idiopathic thrombocytopenia (ITP), preleukemia, acute promyelocytic leukemia, and pregnancy (Hattori et al. 1979) as well as in spur cell anemia secondary to hepatocellular disease (Doll 1982). SEM has been used to study the interaction of platelets with blood borne tumor cells. Some tumor cells are able to activate platelets to change shape and form aggregates in vitro and in vivo (Figure 7). The presence of aggregates distant from the tumor suggests that soluble tumor products in the media may be responsible for the aggregation of platelets. Platelet aggregation by tumor cells in the circulation does not appear to be essential for tumor cell metastasis in the spontaneous metastasis animal model (Estrada, 1983). The morphology of platelet tumor cell interaction has been the subject of a recent review (Warren 1978).

Several basic substances including polylysine, protamine, and polyornithine are strong platelet stimuli which induce shape change and aggregation in vitro. It is therefore of interest that a biologic basic substance, myelin basic protein (MBP), induces dramatic shape changes although no release has been demonstrated (Laubscher et al. 1979).

Contact activation of adherent platelets (Figure 8A-C):

Platelet adhesion is a complex process in which at least 3 partially independent events, cell attachment, morphologic changes (i.e. spreading) and surface induced release, participate (Baumgartner et al. 1976). The morphologic changes which accompany adhesion bear many similarities to those described in aggregating platelets in suspension. These changes can be visualized by light microscopy as platelets react to a glass surface and have been well studied by dark field (Fonio and Schwendener 1942), phase contrast (Bessis and Tabius 1955) and differential interference contrast (DIC) (Allen et al. 1979, Barnhart et al. 1972) microscopy. Bessis and his coworkers characterized the successive stages of platelet shape change, labeling the evolving platelet forms round or discoidal, dendritic, transitional and spread (Bessis and Bricka 1952, Bessis and Burnstein 1949, Bessis and Tabius 1955). More recent studies by Allen and coworkers (1979) combined DIC cinephotography of viable platelets with scanning electron microscopy of fixed platelets to obtain further detail on surface structural changes. They observed early extension of both short nonmotile protuberances and longer cylindrical, tapered pseudopods which extended and retracted until surface contact was made. Platelet cytoplasm (hyalomere) spread radially either symmetrically or asymmetrically. Hyalomere spreading could occur whether or not preceded by pseudopodial activity. When preceded by pseudopods, the hyaloplasm spread as a web connecting adjacent projections. Granules were concentrated





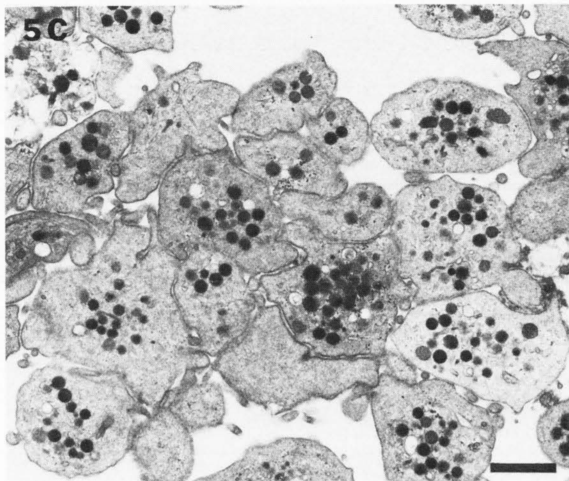


Figure 5 A-C. Ultrastructure of epinephrine induced primary wave aggregates. A and B: SEM and C: TEM. Samples of primary aggregates show that sphering does not accompany primary wave aggregation induced by epinephrine. By SEM (A) aggregates appear composed of plump but discoid platelets. At higher magnification (B) these platelets show a few thin pseudopods but no surface convolutions. TEM (C) of these primary aggregates shows little evidence of centralization of organelles or condensation of cytoplasmic matrix typical of ADP aggregation. Bars = 1 micrometer.

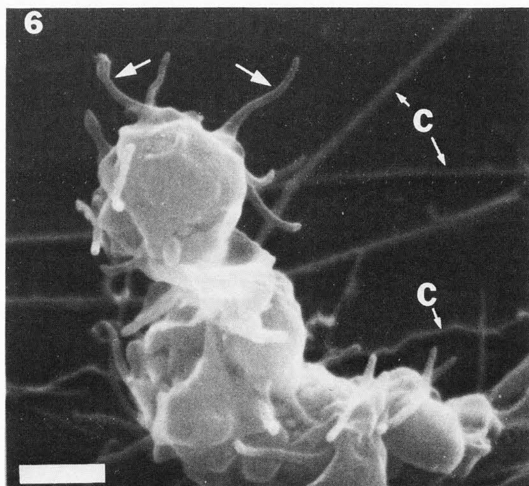


Figure 6. Scanning electron micrograph of collagen induced aggregation. Disc to sphere transformation is accompanied by extensive pseudopod formation (Arrows). Collagen fibrils (C) are present in the background. Bar = 1 micrometer.

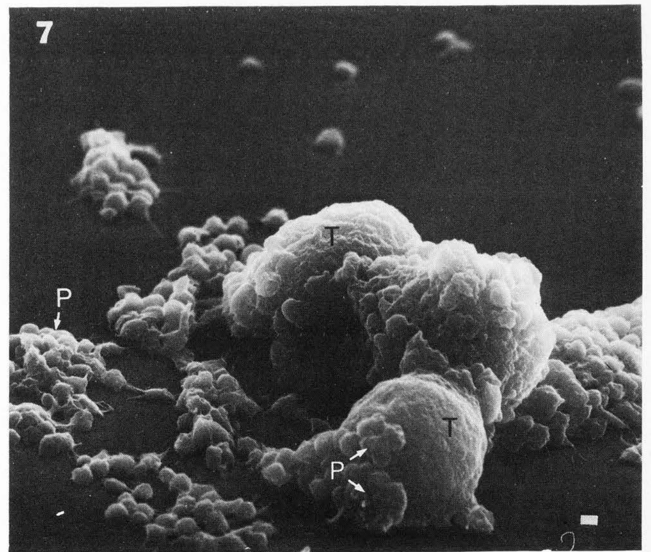
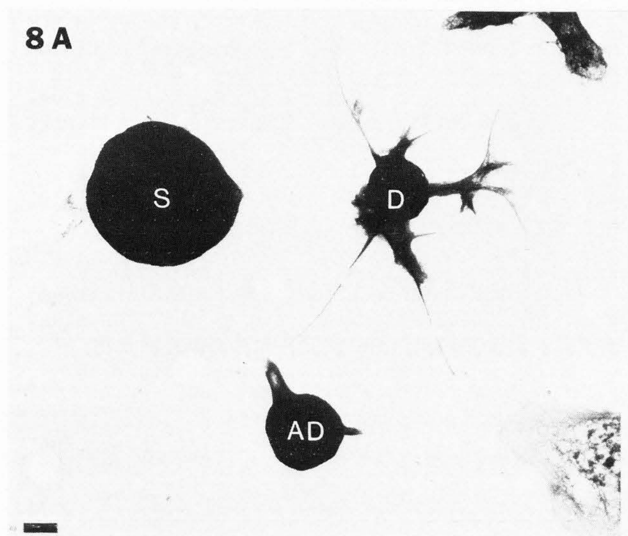
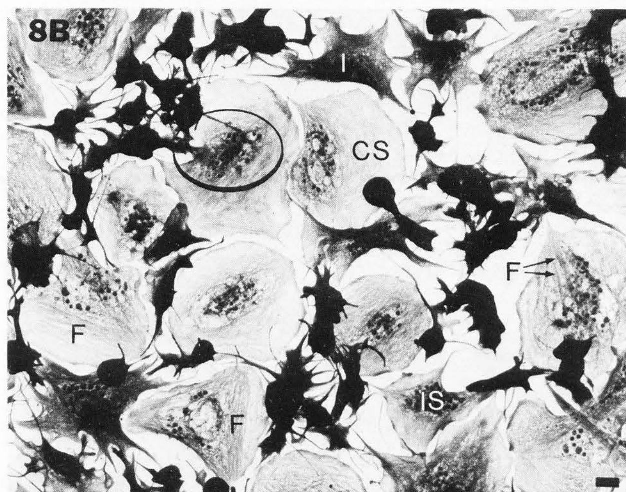
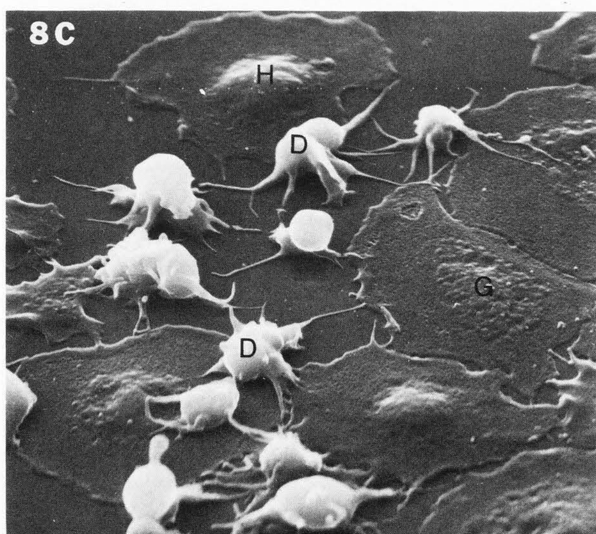


Figure 7. Platelet-tumor cell interaction. Scanning electron micrograph demonstrates the in vitro activation of platelets (P) by tumor cells (T) derived from rat 12763 adenocarcinoma (clone MTF7). Aggregation occurs both with platelets in contact with tumor cells and with platelets distant from tumor cells suggesting that a tumor product in the media promotes aggregation. (Reproduced with permission of J Estrada).







centrally into a hillock reminiscent of the granule centralization observed in platelet aggregates (White 1974a). As spreading proceeded, this hillock flattened. Using mepacrine labeling, these authors were able to observe dense body release. They also observed that platelets settling on top of spread platelets were blocked from the substrate stimulus and did not spread, although they did partially degranulate and formed many pseudopods which interlocked as aggregates formed.

The sequence of morphologic changes on formvar are similar to those observed with glass activated platelets (Lewis et al. 1980, Barnhart and Riddle 1967, Mattson 1981) (Figure 8A-C). Using SEM stereomicroscopy of pigeon thrombocytes adherent to formvar, Taylor and Lewis (1981) extended Allen's observations and demonstrated that attachment of pseudopodia and of the spreading edge of the hyalomere is discontinuous. Stereo whole mount TEM demonstrated fine filament strands connecting the uplifted membrane to the formvar surface. Several investigators (Braunsteiner and Pakesch 1956, Rebuck and Riddle 1964, Riddle and Schatz 1970, Barnhart and Riddle 1967, Barnhart et al. 1970, 1972, and Barnhart and Noonan 1978) have used formvar activation as the basis for a functional assay of platelet reactivity. Platelets in whole blood, stimulated by a timed exposure to formvar can be fixed as whole mounts and examined by either TEM or SEM. Activated forms, similar to those described by Bessis (1973) and Allen et al. (1979) on glass, include round, abortive-dendritic, dendritic, intermediate, and spread forms (Figure 8); these can be quantitated to give an index of reactivity. In addition, aggregrate formation can be quantitated. Hyperactivity, manifested as increased spreading and greater aggregate formation, has been detected in patients with myocardial infarction (MI) in the immediate post

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Figure 8 A-C. Ultrastructure of contact activated platelets. A and B: TEM. C: SEM. Transmission electron micrographs A and B demonstrate whole mount preparations of adherent and spreading platelets on carbon-stabilized formvar films. Extraction-fixation in 2.5% glutaraldehyde containing 1% NP40 allows visualization of cytoskeleton in spread forms. Early forms are seen in A; these include sphered (S), abortive-dendritic (AD), and dendritic (D) forms. In B, later spread forms are demonstrated. As spreading proceeds, intermediate forms (I) show cytoplasmic spreading between pseudopods. Later forms include incompletely (IS) and completely spread (CS) platelets. Centralization of organelles (circle) is clearly evident in spreading platelets, and rearrangement of cytoskeletal filaments (F) can be seen. Scanning electron micrograph C of adherent platelets demonstrates the 3-dimensional appearance of contact activated platelets. Adhesive substrate is a carbon-formvar coated glass coverslip; contact time 60 minutes. Dendritic platelets (D) show numerous thin pseudopods radiating from plump sphered bodies. Spread platelets have centralized granules which first appear as a central hillock (H) and then flatten revealing the outline of individual granules (G). Bars = 1 micrometer.

MI period (100%) as well as several months later (67%) (Riddle and Schatz 1970). Similar increases in reactivity to glass and collagen has been described in ischemic heart disease by Aznar et al. (1979). Hyper-reactivity to formvar has also been seen in patients with arthritis (Riddle et al. 1981) as well as in patients with acute stroke (88%) (Gilroy et al. 1969, Barnhart et al. 1970) but in only 36% of patients with chronic cerebrovascular disease (Walsh et al. 1975). Fibrinolytic products in low concentration enhance platelet contact activation while in high concentrations are inhibitory (Barnhart and Riddle 1967).

This system has also been useful in evaluating platelet reaction to various chemical stimuli. Enhanced platelet activation by biologic amines (serotonin, epinephrine, and norepinephrine), thrombin, collagen, adenine nucleotides, fibrin split products and low concentrations of Hageman factor has been demonstrated (Barnhart et al. 1970, 1972, Barnhart and Riddle 1967, Walsh and Barnhart 1973). In addition these authors have studied inhibition of membrane reactivity by low molecular weight dextran, ouabain, histamine, urea in glucose, and sodium metabisulfite and acetylsalicylic acid (Barnhart et al. 1970, 1972) using SEM as an adjunct to TEM and DIC light microscopy.

The adherence of platelets to biological substrates initiates events similar to those described with glass and formvar activation. Scanning electron microscopy of human platelets adherent to a collagen coated substrate shows a similar sequence of morphologic changes (Elbert et al. 1979). With conversion of discoid platelets to spheres accompanied by a progressive increase over time in bulbous and fine elongated pseudopods, platelet spreading and ultimate aggregate formation developed over a 16 minute time course. These authors reported that PGI_2 did not apparently inhibit adhesion (sticking) to collagen but sphering and pseudopod formation were retarded and aggregates were fewer and smaller. Aspirin (10 mg%) inhibited aggregation but not adhesion on the collagen substrate and morphologic transformation was abnormal with more pleomorphic forms. Sulfinpyrazene (10 mg%) proved least effective as an inhibitor producing no substantial decrease in aggregate size although morphologic alterations were similar to those seen with aspirin.

A role for the adhesive protein fibronectin in platelet-collagen interactions has been postulated (Bensusan et al. 1978). Fibronectin is present in platelet alpha-granules (Zucker et al. 1979, Plow et al. 1979) and is released upon stimulation by thrombin and high concentrations of collagen (Zucker et al. 1979). Fibronectin can be identified on the platelet surface following secretion (Ginsberg et al. 1981) and on spread platelets adherent to collagen fibers or plastic, but not on unstimulated gel-filtered platelets (Lahav and Hynes 1981). Santoro and Cunningham (1979) demonstrated only a slight reduction in adhesion after treatment of washed platelets with anti-fibronectin antibody or Fab fragments. On the other hand, platelet spreading on both collagen and fibrinogen substrates appears to be potentiated by fibronectin (Grinnell et al. 1979). Further support for this is found in SEM studies showing an increase in platelet adhesion to fibronectin-treated fibrillar collagen with marked potentiation of spreading to form a pavement like covering of the substrate (Koteliansky et al. 1981). The role of fibronectin in adhesion is far from clear, but from the data available it may have a major role in supporting platelet spreading with a more minor participation in promotion of the attachment or contact phase of adhesion.

Platelets adherent to glass beads retrieved from glass bead retention columns show the same morphologic transformation with development of dendritic and spread forms as described with glass slides, formvar, and collagen. PGI_2 inhibition of glass bead retention is due to inhibition of aggregation. Platelet attachment appears to be intact but spreading is markedly inhibited (Hattori et al. 1974a,b, Tsao and Krajewski 1982). Inhibition of spreading has been demonstrated by cold (Bessis 1973), cytochalasin B (Taylor and Lewis 1981) and PGI_2 (Tsao and Krajewski 1982). In all instances when these agents or conditions were introduced after spreading had occurred, retraction of spread hyaloplasm left long fine pseudopods behind attached to the substrate.

Cytoskeletal architecture in spreading platelets has been studied using formvar as the activating substrate and partial detergent extraction (Nachmias and Sullender 1978, Nachmias 1980, Mattson and Zuiches 1981b, Mattson 1981), whole mount electron microscopy (Lewis et al. 1980, Taylor and Lewis 1981) and high voltage electron microscopy (Albrecht and Lewis 1982) to visualize the platelet cytoskeleton. Cytoskeletal architecture in detergent extracted platelets is demonstrated in Figure 8B.

Cytoskeletal organization in dendritic platelets shows radial extension of bundles of microfilaments into pseudopods, while in the fully spread platelet there is reorientation of microfilament bundles into circumferential loose parallel arrays (Lewis et al. 1980, Mattson and Zuiches 1981a,b, Mattson 1981, Nachmias and Sullender 1978, Nachmias 1980). A microtrabecular lattice of shorter fine filaments is evident throughout the platelet cytoplasm and there is a condensed filament network in the submembranous cortex (Lewis et al. 1980, Mattson and Zuiches 1981a, b). This reorganization of the platelet cytoskeleton parallels platelet spreading and granule release (Allen et al. 1979). Debus et al. (1981) have identified by immunofluorescence that the circumferential microfilaments are actin and that myosin is concentrated both centrally and at the margin of the spread platelet. An organization of circumferential actin filaments and central concentration of myosin is consistent with the hypothesis that actomyosin interactions result in contraction of actin microfilaments to provide force generation for granule release (Gerrard et al. 1981). Similarly the continuity of radial filaments in pseudopods with the more central circumferential filaments would allow central actomyosin contraction to play a role in pseudopod retraction.

The microtubule coil, important to maintenance

of the discoid shape of the unstimulated platelet, appears to reorganize in the contact-activated platelet but the precise sequence of events is unclear. Using immunofluorescence with anti-tubulin antibodies, Debus et al. (1981) have observed brightly stained microtubular rings with mean maximum diameters of 3.4 μm in discoid platelets. In dendritic platelets these coils decreased in diameter to 2.1 $\pm$ 0.44 μm with one or two arms projecting out from the ring presumably into pseudopods. In contrast Reeber et al. (1983) have observed a gradual breakdown of the microtubule ring during spreading on poly-L-lysine coated surfaces. In these studies anti-tubulin staining became diffuse within the platelet body with bright staining of some pseudopodia. One can see similar diffusely stained dendritic platelets among the spread platelets in photomicrographs taken at 2 hrs by Debus et al. (1981). Whether this diffuse staining represents depolymerization of microtubules is not clear nor is it clear in comparing the two studies whether this phase precedes or follows the formation of a microtubular ring of decreased diameter. Immunofluorescence of more fully spread platelets in 2 hr specimens (Debus et al. 1981) showed various complex arrangements of curving microtubules seen both close to the central granulomere and in the distal spreading cytoplasm suggesting that if depolymerization occurs, reformation of microtubules takes place in later stages of spreading. Ultrastructural studies support the concept of microtubule reorganization during platelet spreading on surfaces (Lewis et al. 1980, 1982, Mattson and Zuiches 1981a,b). Using whole mount stereo electron microscopy of platelets from the African green monkey, Lewis et al. (1982) observed that during the early stages of adhesion, microtubules assumed a radial orientation into the pseudopods of dendritic platelets. As hyalomere spreading proceeded, microtubules paralleled the major axes of the cells until in fully spread platelets the microtubules were present in a diffuse circumferential pattern. Replacement of the coiled microtubule ring of resting platelets with gently curving circumferential microtubules in the cytoplasm of the fully spread platelet has also been observed in human platelets using detergent treated whole mounts (Mattson and Zuiches 1981a,b).

There is evidence that these changes in microtubule organization may not be required for platelet spreading to occur. Lewis et al. (1982) have demonstrated that colchicine and vinblastin treatment of platelets, which dissociates microtubules, does not eliminate spreading although a slight reduction in the percentage of fully spread cells was observed. Similarly, prevention of depolymerization of microtubules using the microtubule stabilizing agent, Taxol, does not interfere with platelet shape change (White and Sauk,1984). One might speculate from the available data that microtubule reorganization occurs as a secondary event during adhesion-associated shape change. A reasonable sequence of events might be: (1) contact activation of the platelet with centralization of granules and a decrease in the microtubule coil diameter resulting in platelet sphering in a manner similar to the initial events seen in aggregating platelets (White 1968d), (2) simultaneous extension of the two ends of the microtubular coil into pseudopods as the dendritic platelet develops, (3) a phase of microtubule depolymerization in the dendritic platelet as cytosol calcium levels rise and (4) reformation of microtubules within the cytoplasm of the spreading platelet as gently curving structures oriented circumferentially both around the central granulomere and throughout the spreading cytoplasm.

Disorders of Platelet Function

This review will limit its discussion to three congenital bleeding disorders chosen because they represent specific defects in the membrane related functions, aggregation and adhesion, and are therefore amenable to SEM study. Indeed, three dimensional structural studies, either SEM or whole mount TEM adhesion assays, of these disorders have contributed significantly more information relative to functional capacity than has conventional TEM of sectioned platelets.

For an in-depth review of the literature on the historic, physiologic, and biochemical aspects of these disorders the reader is referred to several excellent reviews (Lusher and Barnhart 1977, Bloom 1980, Weiss 1980, Barnhart and Lusher 1981, Zimmerman and Ruggeri 1982).

Von Willebrand's Disease (VWD):

In 1926 von Willebrand described 23 members of a family with moderately severe clinical bleeding, prolonged bleeding times and an autosomal pattern of inheritance. Almost 30 years later it was established that an abnormality of Factor VIII was present in this disorder when Alexander and Goldstein (1953) and later Juergens et al. (1957) reported a deficiency of Factor VIII procoagulant activity. As the biochemical and functional characteristics of the Factor VIII molecule have been elucidated, it has become apparent that patients with VWD may express deficiencies in a variety of Factor VIII related functions. In 1971 Zimmerman et al. demonstrated that severe VWD patients were often deficient in both Factor VIII procoagulant activity and Factor VIII related antigen (an antigen identified by a rabbit antibody raised against human Factor VIII) in contrast to hemophiliacs who lack procoagulant activity but possess normal amounts of Factor VIII related antigen. With the discovery of the ability of the antibiotic ristocetin to aggregate platelets in platelet-rich plasma from normal individuals but not from von Willebrand's patients, a biologic assay was developed for quantitating the plasma activity missing in VWD patients (Howard and Firkin 1971). This missing plasma factor in VWD is called von Willebrand factor while ristocetin cofactor activity refers to one functional attribute of von Willebrand factor, i.e. that measured in the ristocetin aggregation assay. In addition to support of ristocetin induced platelet aggregation, von Willebrand factor is required for adhesion of platelets to subendothelium (Tschopp et al. 1974), retention of platelets on glass bead filters (Salzman 1963, Hattori et al. 1974c), and maintenance of a normal bleeding time in vivo.

Defective adhesion to subendothelium has been demonstrated on rabbit aorta (Tschopp et al. 1974), human umbilical vein (Lusher and Barnhart 1977, Barnhart 1978, Barnhart et al. 1981), and human renal artery (Sakariassen et al. 1979). The defect in adhesion to rabbit aortic subendothelium in VWD as demonstrated in an annular perfusion chamber is shear rate dependent (Weiss et al. 1978). The requirement for von Willebrand factor in normal adhesion of platelets in unanticoagulated blood to subendothelium is greatest at shear rates of 2600-3300 sec^{-1}, within the range of those shear rates found in the microvasculature (Weiss et al. 1978, Baumgartner et al. 1977). Baumgartner et al. (1980) have reproduced the adhesion defect of von Willebrand disease using antibodies to human factor VIII/von Willebrand factor.

In their recent review, Zimmerman and Ruggeri (1982) summarize the evidence that Factor VIII is a molecular complex between the small procoagulant molecule and the high molecular weight polymeric von Willebrand factor molecule, and that the von Willebrand factor activity of the Factor VIII complex is dependent on the presence of high molecular weight multimers (Ruggeri and Zimmerman 1980, 1981).

Classification of subtypes of VWD is based on differentiation of abnormalities of von Willebrand factor in plasma and platelets as well as on identification of variations in the mode of genetic transmission. The more common types of VWD (Types I, IIA, and IIB) show an autosomal dominant inheritance. Type I VWD, the most common subtype, is the result of a quantitative defect in the amount of von Willebrand factor present in plasma and is secondary to an absolute decrease in the amount of immunologically detectable factor VIII antigen. Multimer formation is normal. Because the absolute quantity of antigen is decreased, there is a proportional decrease in ristocetin cofactor activity and in the amount of factor VIII procoagulant activity. The latter is presumably secondary to lack of available von Willebrand factor for complexing and carrying the procoagulant molecule. Platelet von Willebrand factor is normal in quantity and multimer formation (Ruggeri et al. 1978, Zimmerman and Ruggeri 1982). Type II VWD is characterized by an absence of large multimers of von Willebrand factor in plasma. In type IIA large multimers are absent from both plasma and platelets (Ruggeri and Zimmerman 1981, Zimmerman and Ruggeri 1982) and this absence is thought to reflect a defect in multimer assembly (Zimmerman and Ruggeri 1982). In type IIB , high molecular weight multimers are absent from plasma but present in platelets (Ruggeri and Zimmerman 1980, 1981). The von Willebrand factor of type IIB is hyperresponsive to ristocetin leading to the hypothesis that the decrease in plasma multimers is secondary to increased binding of an abnormally avid von Willebrand factor to platelets and tissue sites (Zimmerman and Ruggeri 1982). Type III VWD is the rarest type. This group contains the autosomal recessive forms of VWD. Affected individuals are severe bleeders and von Willebrand factor levels in homozygotes and double heterozygotes may be undetectable in plasma, platelets, or endothelial cells, suggesting a defect in synthesis or degradation within the cell of origin prior to release.

"Pseudo" von Willebrand's disease has been recently described (Gralnick et al. 1981, Weiss et al. 1982). In this disorder, which resembles type IIB VWD, a platelet abnormality is responsible for enhanced von Willebrand factor-platelet interaction following ristocetin stimulation. The abnormal platelets bind normal von Willebrand factor at lower concentrations of ristocetin than required to initiate von Willebrand factor binding to normal platelets. In one instance, von Willebrand factor induced platelet aggregation without ristocetin. The absence of large multimers in the plasma but not in the platelets in this disorder is probably due to enhanced removal by binding to platelets (Zimmerman and Ruggeri 1982).

Conventional ultrastructural studies on ultrathin sections have not revealed any diagnostic morphologic alterations in VWD platelets. While several authors (Schulz et al. 1958, Jean et al. 1963, Marx and Jean 1964, Boisseau et al. 1976) have described abnormal drumstick granules in platelets of patients with von Willebrand's disease, Hovig (1968) and Lusher and Barnhart (1977), in their reviews of platelet morphology in disease, point out that this ultrastructural finding has been observed in other pathologic conditions (Jean et al. 1963, Marx and Jean 1964, Seip and Kjaerheim 1965) and in normal dog and human platelets (Hovig 1968). Lusher and Barnhart (1977) suggest that morphometric analysis would provide quantitative data on the frequency of these granules in VWD vs. normal platelets. Platelets from swine with VWD show no abnormalities by SEM or TEM; in addition, they are capable of normal uptake of latex particles (Lewis and Bowie 1978).

Scanning electron microscopy has yielded important data on the responsiveness of VWD platelets to foreign and biologic surfaces. By SEM, reduced membrane responsiveness to formvar in a non-flow system has been observed in VWD platelets (Lusher and Barnhart 1977, Barnhart 1978) although adhesion itself was described as fair in this test system. Altered surface responsiveness was reflected in failure of disc to sphere transformation, defective pseudopod formation with a decrease in fully developed dendritic platelets, and increased numbers of abnormal spread forms (Lusher and Barnhart 1977).

In a similar manner, defective adhesion of VWD platelets to subendothelium has been studied ultrastructurally by SEM. Using an umbilical vein ex vivo perfusion model, Barnhart and her associates (Lusher and Barnhart 1977, Barnhart 1978, Barnhart et al. 1981) demonstrated uniformly decreased adhesion of VWD platelets ranging from 7-51% of normal. While the majority of the VWD patients tested showed decreased spreading of platelets in addition to decreased adhesion, two patients demonstrated normal spreading over the exposed subendothelium. Bolhuis et al. (1981) reported that binding of von Willebrand factor to subendothelium precedes platelet adhesion and spreading. Sixma et al. (1982a), using human artery subendothelium in an annular perfusion chamber, have demonstrated a greater degree of impaired spreading in type IIA,

IIB, and III VWD as compared to type I. The difference in reactivity did not correlate with von Willebrand factor levels as reflected in the ristocetin cofactor activity assay. Turitto et al. (1983) eliminated the contribution of spreading to the adhesive process by chelating cations with EDTA and demonstrated a reduced attachment phase to chymotrypsin-treated subendothelium in VWD as compared to normal. These findings suggest a role for high molecular weight multimers of von Willebrand factor in both attachment and spreading on subendothelium at appropriate shear rates.

It appears well established that one can no longer envision adhesion as a simple process; adhesion clearly involves multiple complex steps including attachment, cellular activation, granule release and spreading (Allen et al. 1979, Turitto and Baumgartner 1982). While our understanding of the role of von Willebrand factor in adhesion to subendothelium has grown enormously in the past ten years, the factors which modulate the subsequent activation steps such as granule release and spreading are not yet worked out. Scanning electron microscopy is becoming an increasingly important tool in evaluating mechanisms which control platelet activation and spreading on surfaces.

The Bernard Soulier Syndrome (BSS):

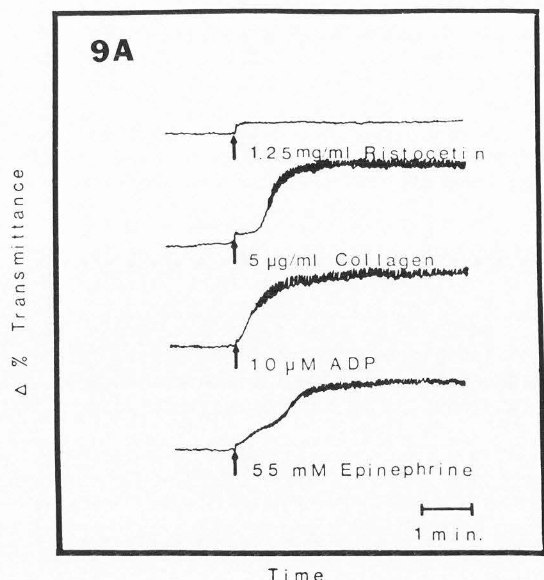
The Bernard Soulier Syndrome (BSS) is an autosomal recessive bleeding disorder characterized by variable degrees of thrombocytopenia, a prolonged bleeding time, and giant platelets on peripheral smear (Bernard and Soulier 1948). The bleeding time and clinical symptoms are out of proportion to the thrombocytopenia suggesting an intrinsic platelet defect (Caen et al. 1976). Additional laboratory characteristics include a normal clot retraction, defective prothrombin consumption, and diminished binding of coagulation factors V, VIII and XI to BSS platelets (Grottum and Solum 1969, Walsh et al. 1975). Aggregation of BSS platelets with ADP, epinephrine, and collagen is normal (Bithell et al. 1972, Weiss et al. 1974, Caen et al. 1976). Indeed, the rate of ADP-induced aggregation has been reported to be accelerated (Bithell et al. 1972). However, BSS platelets are not aggregated by ristocetin (Howard et al. 1973a, Caen and Levy-Toledano 1973) (Figure 9A) or bovine factor VIII (Weiss et al. 1974, Caen et al. 1973, 1976). In contrast to von Willebrand's disease where defective ristocetin aggregation is corrected by replacing the missing von Willebrand factor, normal plasma containing von Willebrand factor does not correct the defective ristocetin aggregation of BSS (Caen and Levy-Toledano 1973, Howard et al. 1973b, Firkin et al. 1974). Based on these observations, it has been suggested that a membrane receptor for the von Willebrand factor might be missing in BSS platelets. Grottum and Solum (1969) were the first to describe a platelet membrane defect in BSS when they demonstrated altered electrophoretic mobility and decreased membrane sialic acid content. The decreased sialic acid may be responsible for the shortened survival of BSS platelets (S Murphy 1972). Nurden and Caen (1975) extended these observations demonstrating deficient PAS staining of the glycoprotein I complex on SDS-PAGE gels of isolated BSS platelet membranes. Nachman et al. (1977) suggested that glycoprotein I (GP I) complex mediates ristocetin aggregation. Nurden and Caen (1976, 1977), George et al. (1981), and Jamieson et al. (1979a) subsequently confirmed the defect in GP I complex in BSS platelets. The resolution of GP I complex into its component glycoproteins by two dimensional gel electrophoresis (Nurden et al. 1981, Peterson 1982) has allowed specific identification of GP Ib (Mr 139,000. pI 6.36) as the missing membrane component of BSS platelets. Glycocalycin (GP Is), a macropolypeptide associated with the GP I complex, has also been reported absent from BSS platelets (Jamieson et al. 1979b). Thus, a relationship between glycocalycin and the missing GP Ib has been suggested (Hagen et al. 1980). An antibody isolated from a transfused BSS patient inhibited ristocetin and bovine factor VIII aggregation of normal platelets as well as platelet adhesion to subendothelium (Tobelem et al. 1976). In addition, immunoprecipitation of platelet membrane proteins with this antibody brought down a 155,000 molecular weight component (Degos et al. 1977) providing further evidence that the membrane glycoprotein GP Ib missing in BSS is required for von Willebrand factor mediated functions.

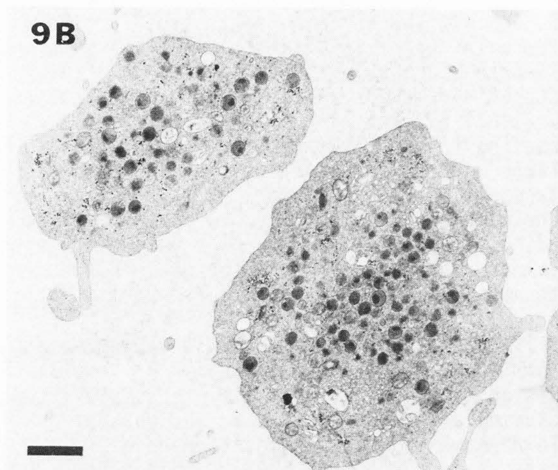
While reports on the BSS platelet response to thrombin have been inconsistent, at least two laboratories (Ganguly 1977, Jamieson and Okumura 1978) have demonstrated reduced numbers of thrombin binding sites and defective thrombin aggregation. Okumura et al. (1978) postulate that thrombin binds to a specific sequence in GP Is. Recently Nurden and Dupuis (1981) have reported the absence of a band corresponding to GP V. This required labelling techniques that were more sensitive than those previously used to detect GP I abnormalities. The lack of GP V may be responsible for the altered response to thrombin reported in some BSS patients as this glycoprotein is a substrate for thrombin. Clementson et al. (1982) have confirmed the absence of GP Ib and GP V and have in addition demonstrated a deficiency of a low molecular weight glycoprotein, GP 17.

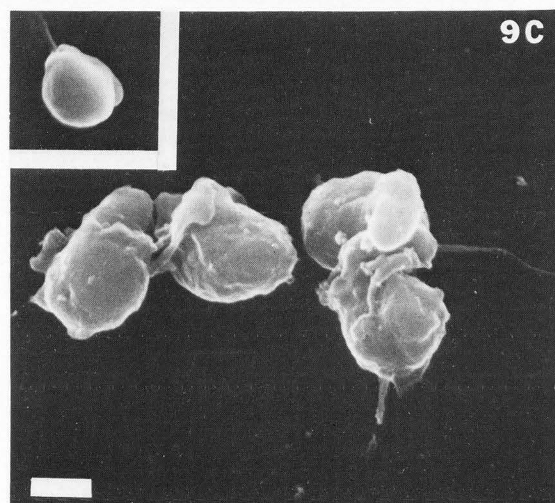
Kunicki and Aster (1978) using a ^{51}Cr-lysis assay found that BSS platelets were not destroyed by quinine/quinidine dependent antibodies, and postulated that the receptor for these drug-antibody complexes is deleted in BSS platelets. Van Leeuwen et al. (1982) using a platelet suspension immunofluorescence test demonstrated that drug induced antibodies require the Fc portion of the molecule to bind to platelets. They further showed that although these antigen-antibody complexes didn't produce lysis of BSS platelets, some of the antibodies did bind to BSS platelets while others didn't. This suggests that the Fc receptor for drug dependent antibodies is not necessarily absent in BSS platelets but may be altered in such a way as to prevent subsequent lysis.

The major functional abnormality in BSS is a defect in adhesion. Adhesion to rabbit subendothelium is defective in both VWD (Tschopp et al. 1974) and BSS (Weiss et al. 1973, 1978, Caen et al. 1976); Weiss et al. (1974, 1978) propose that the initial contact or attachment phase of adhesion is defective in BSS. Zucker et al. (1977) and Moake et al. (1980) have demonstrated defec-

Platelet Aggregation in BSS







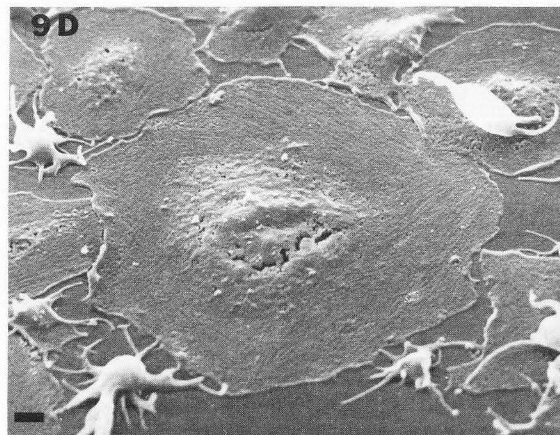


Figure 9 A-D. Bernard Soulier Syndrome (BSS). A: Typical aggregation patterns. B: TEM of sectioned BSS platelets. C: SEM of EDTA-anticoagulated BSS platelets in suspension. D: SEM of adherent spread platelets from BSS heterozygote on carbon-formvar surface; contact time 60 min. Aggregation curves in A demonstrate characteristic isolated absence of ristocetin aggregation. By conventional TEM (B) of sectioned BSS platelets, the only abnormality noted consistently is the presence of large platelets with a corresponding increase in normal appearing granules. By SEM, EDTA-anticoagulated BSS platelets in suspension (C) are enlarged as compared to normal platelets (insert). Note that EDTA induces sphering of both BSS and normal platelets. SEM of adherent and spreading platelets from BSS heterozygote (the mother of 2 affected siblings) shows the giant size of occasional platelets interspersed among normal sized platelets. The small tear in the membrane near the granulomere is an artefact. Eleven percent of this BSS heterozygote's platelets were enlarged (>3.5 µm) by light microscopy. In addition, 10% of her spread forms were large (>12.5 µm). Bars = 1 micrometer.

tive binding of radiolabelled von Willebrand factor to BSS platelets. These data suggest that, under the appropriate flow conditions, adhesion of platelets to subendothelial structures is mediated by von Willebrand factor via membrane glycoprotein Ib/Is.

Morphologic studies are of particular interest in BSS, since giant platelets on smear are a diagnostic feature of the disorder (Figure 9A-D). Mean platelet diameters as measured on peripheral smears demonstrate consistently enlarged platelets in several reported kindred: 3.2-3.4 µm (N=1.83±0.16) (Weiss et al. 1974), 3.09-4.44 µm (N=2.13±0.02) (George et al. 1981) and 3.5-3.7 µm (N=1.5-2.4) (Bithell et al. 1972).

In contrast, Frojmovic et al. (1977, 1978) have measured BSS discoid platelets and found them to be of near normal size. These authors found that induction of sphering produced BSS platelets that were disproportionately larger than normal sphered platelets (Frojmovic and Milton 1982) (Figure 9C). They concluded that a hypervolumetric shape change occurs following stimulation and that the giant appearance of BSS platelets in smears reflects abnormal membrane behavior during preparation of blood films (Frojmovic and Milton 1982). Evagination of an excessive internal membrane system has been suggested as a mechanism. Lusher and Barnhart (1977), however, found markedly enlarged BSS platelets by TEM, and we have made similar observations on BSS platelets in directly fixed buffy coats (unpublished observations). Other evidence of altered membrane reactivity has been reported by Bithell et al. (1972) and Walsh et al. (1975) who demonstrated absent ADP-induced shape change in some of their BSS patients; this finding has not been confirmed in other affected kindred (Caen et al. 1973, Howard et al. 1973a). However, Caen et al. (1973) demonstrated that shape change takes longer to occur in BSS platelets which may explain the divergent observations on its presence by other investigators.

Ultrastructural studies have described a variety of inconstant changes including disorganized microtubules, granule deficiencies, prominence of the OCS and DTS and increased vacuoles. Some platelets have been described as having a Swiss cheese appearance. In general, however, there are no characteristic ultrastructural features of the BSS platelet revealed by transmission electron microscopy other than the large size and a proportional increase in granules (White 1982, Maldonado et al. 1975, Mattson et al. 1983) (Figure 9B). Chevalier et al. (1979) have demonstrated an abnormality in distribution of membrane associated particles (MAPS) as visualized in replicas of freeze fractured platelets. Rather than an equal and random distribution of MAPS on both exoplasmic (E) and protoplasmic (P) faces of the bilipid membrane as seen in the normal, the BSS platelet had a greater concentration of particles on the protoplasmic face. We have recently reported that BSS platelets in native plasma are normally reactive to a formvar surface in a non-flow system, demonstrating a rate of spreading comparable to or slightly greater than that of controls (Mattson et al. 1984). In addition fully spread platelets from both homozygotes and heterozygotes demonstrated heterogeneity of size with giant spread forms representing 22-44% of homozygote platelets and 0-10% of heterozygote platelets (Figure 9D). Despite the abnormal size of a large percentage of BSS spread platelets, cytoskeletal architecture in both large and normal sized BSS platelets, as revealed by detergent extraction of whole mounts for TEM, was identical to normal-sized control platelets.

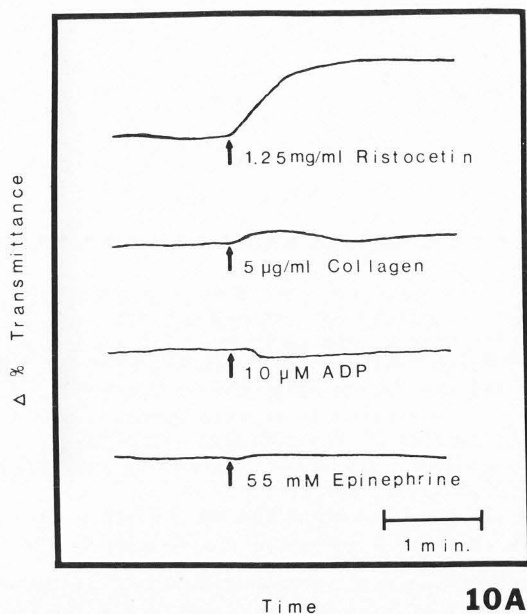
Glanzmann's Thrombasthenia (GT):

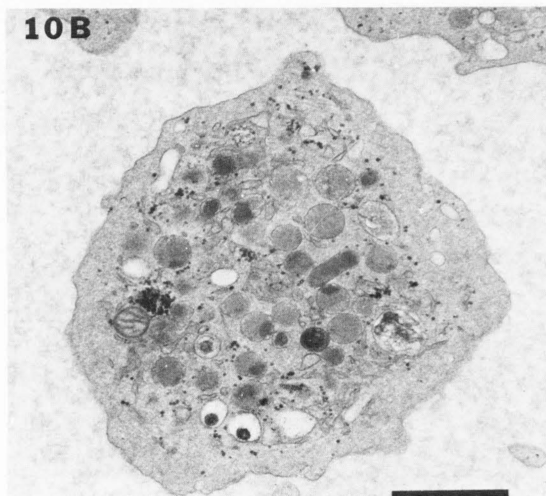
Thrombasthenia is a rare disorder of platelet function first described by Glanzmann in 1918. It now appears that the original patients were a heterogeneous group who had in common normal or near normal platelet counts but defective clot retraction. Naegeli (1931) and Fonio and Schwendener (1942) demonstrated that a prolonged bleeding time and isolated rather than aggregated platelets on peripheral smear were further characteristics of this disorder. The main functional defect is an impairment of platelet aggregation (Weiss 1975). Primary wave aggregation is absent in response to ADP, epinephrine, collagen, serotonin, and noradrenalin (Hardisty et al. 1964, Caen et al. 1966, 1972, Lusher et al. 1977) (Figure 10A).

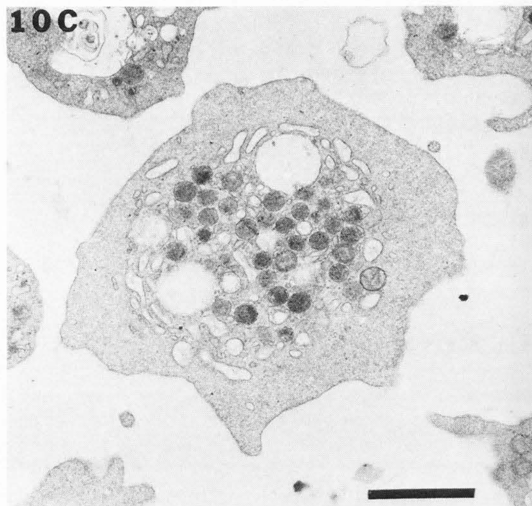
Early studies on the pathophysiology of Glanzmann's thrombasthenia (GT) implicated deficiencies in various platelet enzymes (Gross 1961, Karpatkin and Weiss 1972, Moser et al. 1968) but consistent abnormalities have not been reported. Marcus and Zucker-Franklin (1964) and Caen et al. (1966) independently suggested that a primary membrane abnormality was responsible for the aggregation defect seen in these patients. However, direct evidence for such a defect was not found until the seventies when a decrease or absence of the externally exposed membrane glycoprotein complex GP IIb-IIIa was described by Nurden and Caen (1974) and confirmed by Phillips et al. (1975, 1977) and Peterson and Wehring (1981). It has been demonstrated that thrombasthenic platelets frequently lack fibrinogen (Caen et al. 1966, Nachman 1966, Nachman and Marcus 1968) and fail to bind fibrinogen normally following stimulation by ADP (Pfueller and Firkin 1978, Bennett and Vilaire 1979), suggesting that the fibrinogen receptor may be GP IIb-IIIa. Complexing of GP IIb-IIIa requires calcium (Jennings and Phillips 1982, Pidard et al. 1982). McEver et al. (1983), using a monoclonal antibody with specificity for the IIb-IIIa complex but not to either IIb or IIIa alone, have demonstrated equal antibody binding to resting and stimulated platelets. This suggests that IIb and IIIa do not exist as discrete and separate glycoproteins in the resting platelet membrane but are complexed in both the resting and stimulated platelet. The binding of fibrinogen mediates aggregation by a mechanism not as yet worked out although various possibilities including direct bridging of adjacent platelets by fibrinogen have been suggested.

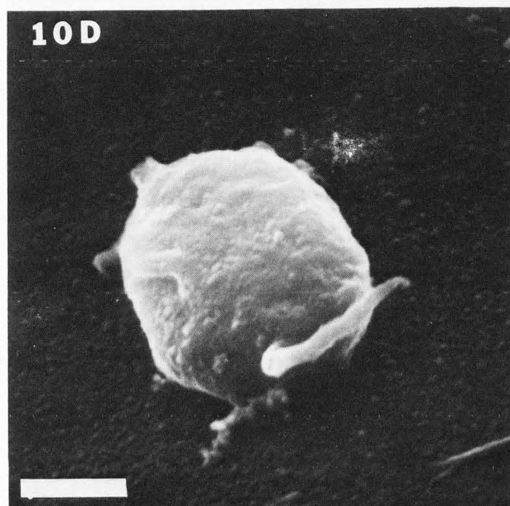
In addition to lack of fibrinogen, deficiency of alpha-actinin has been detected in GT patients (Gerrard et al. 1979) suggesting that the GP IIb-IIIa complex may be bound to the platelet cytoskeleton via this protein. In addition, the platelet antigen Pl^{A1} has been shown to be associated with GP IIIa (Kunicki and Aster 1979) and patients with thrombasthenia have variable deficiencies in Pl^{A1} (Kunicki and Aster 1978). De Gaetano et al. (1977) suggest from data on defective GT fibroblast retraction in culture that the abnormality in cellular binding of fibrin/fibrinogen might not be limited to platelets and might be responsible for the defective late wound healing observed at the skin biopsy site in their donor GT patient. More recently normal monocyte membranes have been reported to contain GP IIb-IIIa but no data on monocytes from GT patients was reported (Gogstad et al. 1983). No comparable defect in red cell glycoproteins has been detected (George et al. 1980).

Glanzmann's thrombasthenia has been recognized in both sexes. Based on a lack of significant clinical symptoms in parents of affected individuals and the frequency of consanguinity in some









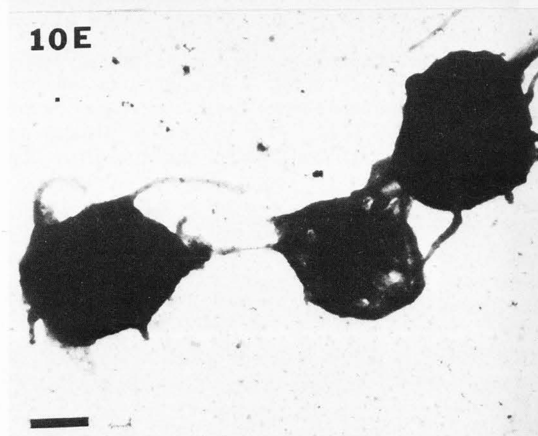


Figure 10 A-E. Glanzmann's thrombasthenia (GT). A: aggregation patterns. B and C: Transmission electron micrographs of resting and ADP stimulated GT platelets respectively. D: Scanning electron micrograph of adherent GT platelets on a carbon-formvar surface; contact time 60 min. E: TEM of whole mount preparation of adherent GT platelets on carbon-formvar; contact time 60 min. In A the characteristic absence of aggregation in response to ADP, collagen, and epinephrine is seen. TEM of unstimulated GT platelets show no abnormalities (B). ADP stimulated GT platelets (C) are able to undergo shape change (sphering and centralization of granules) in response to 2×10^{-6} M ADP despite inability to aggregate. In contrast, GT platelets do not spread normally on a carbon-formvar surface (D and E). By SEM (D) and TEM (E), pseudopod formation is absent or abortive and spreading is not seen. Bars = 1 micrometer.

of the described affected families, an autosomal recessive inheritance has been proposed (Caen et al. 1966).

Bleeding, which varies in severity and can be life threatening, usually begins in early life but tends to decrease with age. The bleeding is characterized by mucosal and cutaneous purpura. The diagnostic laboratory features are a normal platelet count, prolonged bleeding time, dimi-

nished or absent clot retraction and markedly diminished primary wave aggregation (Weiss 1975). Regardless of the method used, aggregation by ADP, epinephrine, serotonin (5-hydroxytryptamine), thrombin, and collagen is defective (Hardisty et al. 1964, Caen et al. 1966, 1970, 1972, Zucker et al. 1966, Cronberg et al. 1967, 1971). Thrombasthenic platelets aggregate in response to ristocetin in the presence of adequate factor VIII and to bovine fibrinogen (presumably due to the presence of bovine factor VIII) (Weiss 1975). Despite the inability to aggregate to all concentrations of ADP, thrombasthenic platelet membranes bear the ADP receptor (Bennett et al. 1977) and demonstrate the ability to change shape following ADP stimulation (Zucker et al. 1966) (Figure 10C). Platelet release of ADP and PF4 are normal in response to collagen and thrombin (Kubisz et al. 1971, Caen 1972, Weiss and Rogers 1973) but defective in the presence of ADP and epinephrine (Zucker et al. 1966, Caen et al. 1970, Cronberg et al. 1967). While the aggregation defect appears well established, thrombasthenic platelets show variable defects in adhesion depending on the method and substrate tested. In vivo platelet adhesion, as measured by the method of Borchgrevink, is decreased (Borchgrevink 1961). Adhesion of thrombasthenic platelets to collagen has been reported as normal or near normal (Balleisen et al. 1977, Caen 1972, Zucker et al. 1966) in some patients while defective interaction with collagen has been reported in other GT patients (Balleisen et al. 1977), with abnormal adhesion, defective pseudopod formation and absent spreading. Adhesion of GT platelets to subendothelium and to chymotrypsin-exposed collagen fibrils is reduced with absent platelet thrombi. Examination of adherent GT platelets by TEM demonstrated centralization of filaments, sphering, and decrease in granules suggesting that the contractile wave and release had occurred (Baumgartner et al. 1977). Platelet retention in a glass bead column is often markedly reduced (Salzman 1963); this has been attributed to the defect in aggregation since this test assesses both adhesion and aggregation. Platelet adhesion and spreading on a glass slide is absent or markedly reduced (Caen et al. 1966, 1972) as is adhesion to formvar in a non-flow system (Lusher and Barnhart 1977, Lusher et al. 1977).

By light microscopy of stained smears the only defect appreciated is the lack of aggregation; no abnormality has been noted in individual platelets. Electron microscopy of thin sectioned unstimulated GT platelets reveals essentially normal ultrastructure (Lusher and Barnhart 1977, Boisseau et al. 1976, Caen et al. 1966) (Figure 10B). In ADP stimulated GT platelets, a contractile wave can be appreciated as granules and microfilaments condense centrally. In contrast to normal ADP aggregates in which each compact granule/microfilament mass is oriented toward the center of the aggregate, in GT platelets the contractile wave is oriented to the center of each unaggregated platelet (Gerrard et al. 1979) (Figure 10C).

Morphologic abnormalities in adhering GT platelets have been described using a variety of techniques. Rosenstein et al. (1981) have demonstrated defective spreading on a siliconized glass slide by DIC microscopy. Failure to interact with fibrin strands was also noted. Lusher and colleagues (Lusher and Barnhart 1977, Lusher et al. 1977) using contact activation and adhesion to formvar to evaluate platelet membrane reactivity and viewing the reacting platelets by either SEM or TEM have demonstrated a decrease in adhesion after 8 minutes of exposure to formvar. Pseudopod formation was abnormal and subsequent spreading defective (Lusher and Barnhart 1977, Lusher et al. 1977). Adherent platelets demonstrated sphering with convoluted surface contours. In a similar test system using an extended period of exposure to the formvar surface, we have also observed decreased numbers of attached platelets, defective pseudopod formation and total absence of spread forms even after 60 minutes of contact (Figure 10D,E).

The defective adhesion and spreading on foreign surfaces demonstrated in GT platelets in non-flow systems suggest a role for the missing GP IIb-IIIa in platelet attachment and spreading. This possibility is further supported by observations that fibrinogen coating of substrates enhances platelet adherence (Packham et al. 1969, Zucker and Vroman 1969). Since GP IIb-IIIa is the putative fibrinogen receptor, platelet adhesion to foreign surfaces may be mediated at least in part through GP IIb-IIIa interaction with a fibrinogen coat. It must also be considered that the defect in spreading may not be directly related to the missing membrane glycoproteins but may be the result of defective adhesion-induced release of cofactors required for spreading.

New Directions

The importance of platelet membrane receptors in adhesion and aggregation reactions both as binding sites for agonists that initiate the platelet response and as final effector sites for binding to molecules that directly mediate adhesion and/or aggregation cannot be denied. SEM has played a major role in increasing our understanding of membrane events during platelet activation. Use of specific labeling techniques with visible probes has proved an exciting recent development for study of receptor mobility, orientation, and relationship to localized subcellular structures such as membrane systems (OCS, DTS), and cytoskeletal components. Nurden et al. (1980) used gold spheres bound to lectins to demonstrate lectin binding sites on washed, fixed, discoid (resting) platelets. Wheat germ agglutinin (WGA) receptors were seen scattered over the platelet surface and in the rare pseudopod present. Concanavalin A (conA) binding could be seen by TEM but not SEM. The difference in SEM/TEM visualization is unexplained; the authors suggest that perhaps conA receptors in the resting platelet are in cryptic sites inaccessible to Au particles of 12-50 Å size. SEM showed an even distribution of bound WGA, and by double labeling TEM studies with gold particles of two different sizes, WGA and conA distributions were seen to be separate. These immunolabelling studies correlate with biochemical

data demonstrating that conA binds to a major membrane GP of 100,000 molecular weight (Nachman et al. 1973) while WGA binds to 2 different glycoproteins of molecular weights 150,000 and 210,000 respectively (Nachman et al. 1977).

While this study specifically restricted itself to the unstimulated platelet, other surface labelling studies have examined receptor mobility following stimulation. Furlan et al. (1981) examined the distribution of colloidal gold complexed to low molecular weight oligomers of bovine factor VIII on platelet surfaces using both SEM and TEM. On unfixed platelets the gold-factor VIII probe was capable of inducing aggregation, and the stimulated platelets showed random distribution of the probe on the surface with greatest binding to platelets that appeared stimulated or damaged by TEM. If platelets were fixed first, factor VIII binding appeared most concentrated on pseudopods. This work suggests receptor site redistribution with stimulation. In a slightly different approach, Polley et al. (1981) have used immunoelectron microscopy with ferritin and hemocyanin labels on intact platelets followed by lysis and examination of resultant isolated membranes by TEM to examine membrane glycoprotein distribution in unstimulated and thrombin-stimulated platelets. They found random distribution of both GP IIb and GP IIIa in unstimulated platelets while GP Ib was present in small clusters. Thrombin stimulation produced large mixed clumps of GP IIb and GP IIIa and separate large clusters of GP Ib.

Barnhart and Robinson (1975) used latex probes coupled with antibodies to platelet factor 3 (PF3) and fibrinogen to demonstrate their distribution before and after thrombin stimulation. By this technique thrombin activated platelets demonstrated significantly more PF3 in the membrane surface than unactivated platelets. Fibrinogen was slightly increased on the platelet surface after thrombin stimulation but more important was the observation that fibrinogen was concentrated at the platelet periphery and along pseudopods rather than randomly dispersed. In more recent studies, using an antibody to factor VIII antigen covalently linked to a latex probe, Lusher and Barnhart (1977) demonstrated by SEM that less binding occurs to VWD platelets than to normal platelets, suggesting a decrease in surface-bound factor VIII on VWD platelets.

Albrecht and Lewis (1982) presented work on mapping contact activation-induced changes in membrane receptors for fibrinogen, fibronectin and thrombospondin using gold-protein beads and gold immunobeads.

This new approach will allow investigators to use SEM to identify functional defects in receptors in patients who have antigenically normal amounts of receptor present. With the development of monoclonal antibodies to mediator proteins such as VWF and fibronectin and to specific platelet membrane components, the future will surely see the "mapping" of platelet functional sites.

Conclusion

While the direct examination of platelets by SEM and TEM may be helpful in determining if platelets have been activated in vivo, the information derived is limited and does not allow one to distinguish etiologies. A more important application of correlative SEM/TEM in the diagnosis of platelet dysfunction is the observation of platelet reactivity to aggregating agents or activating surfaces. Under these circumstances SEM and TEM can serve as important adjuncts to the clinical assessment of platelet function. It must be emphasized that because three dimensional changes in form may be the result of several different internal events, maximum information is obtained when SEM and TEM are used together. Using correlative SEM/TEM, the ability of platelets to undergo the normal physiologic responses of adhesion, spreading, pseudopod formation, granule release, aggregation, and internal contraction can be assessed ultrastructurally. With the use of visible probes, future mapping of membrane receptors for agonists and inhibitors and membrane sites mediating adhesion and aggregation will undoubtedly be added to our diagnostic capabilities and will allow more precise documentation of those platelet disorders associated with acquired and inherited membrane abnormalities.

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Discussion with Reviewers

D.A. Walz: One of the major unanswered questions in platelet release reactions is the process of degranulation. Have you any SEM or TEM data which readily supports one type of mechanism, say granule membrane fusion, over alternative hypotheses, such as granule membrane disintegration or release?
Author: To my knowledge, there is no morphologic data in the literature which definitively distinguishes between platelet granule release by a mechanism of membrane fusion versus a mechanism of membrane dissolution. However, it seems likely, drawing parallels from other secretory cells, that the process probably includes fusion of granule membranes with membranes of the open canalicular system (OCS) with subsequent rupture of the fused membranes and release of granule contents into the OCS. In support of this thesis, White (1973) has demonstrated granule contents within the OCS following poly-L-lysine stimulation of platelets.

D.A. Walz: You have made reference to the process of contractile wave observations (Figure 4). Could you elaborate on its definition and description; is it a physical process observed by SEM? The term implies one of continual motion, particularly directional motion, and I am unclear regarding its relationship to SEM.
Author: Morphologic data supporting the concept of a contractile wave comes primarily from TEM observations of sectioned unstimulated and stimulated platelets. Stimulus-induced shape change is accompanied by the appearance of a dense central matrix of contractile filaments. When aggregates are formed, these masses of filaments are oriented toward the center of the aggregate rather than the center of each participating platelet, but when aggregation is blocked, as it is in Glanzmann's thrombasthenia, the filament matrix is found in the center of each individual platelet. Accompanying this condensation of cytoskeletal filaments is the migration of platelet granules to the cell center. It is implied that the forces for granule movement and their subsequent secretion is generated by the contraction of actomyosin within the cytoskeleton. Further morphologic data for contraction of the cytoskeleton comes from studies of isolated cytoskeletons from thrombin-treated platelets. Jennings et al. (1981) observed that the shell of actin filaments, which forms around the centralized granules in thrombin stimulated platelets, contracts from a diameter of 2.1 µm to 1.5 µm.

D.A. Walz: You make reference to seeing similar structures by SEM and TEM. Is it possible to go from one procedure to the other on the same specimen or is the reference more general in that it refers to similar regions in different platelets?
Author: It is possible to examine whole mount preparations of adherent platelets first by TEM and then, after appropriate coating with a highly conductive element such as gold, to examine the identical cells by SEM. The use of "finder grids" simplifies identification of specific cells. However, in the correlative SEM/TEM studies illustrated in this review which demonstrate the morphologic changes acompanying platelet aggregation (Figures 4 and 5), it was necessary to examine separate aliquots from the same aggregometer sample. This was necessary because the TEM samples were embedded and sectioned, while the SEM samples were of whole cells. Our method of preparing aliquots of the same sample for SEM and TEM have been previously published in detail (Mattson et al. 1977).

D.A. Walz: One of the most exciting extensions of SEM is its application to platelet mapping. Do you envision these procedures as ultimately being capable of resolving specific receptor sites, such as those for thrombin and collagen?
Author: Absolutely. With the use of probes visible at the resolving power of SEM, such as colloidal gold, studies can be performed with specific proteins such as collagen or thrombin directly bound to the probe and the pattern of attachment observed. Such studies have already been reported for fibrinogen, fibronectin and factor VIII (see text). Conversely, putative receptors can be mapped using specific antibodies to individual membrane glycoproteins. This approach will be invaluable in the investigation of the mechanisms by which receptors modulating adhesion and aggregation become available after stimulation of the platelet by specific agonists. In the area of diagnostics, it will allow rapid identification of missing membrane receptors.