Fundamentals of clinical cardiology

Platelets and thrombogenesis—Current concepts

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Anticoagulants, although effective in the treatment of venous thrombotic disease, have not been generally helpful in preventing arterial thrombosis. The reason for this disparity may lie in the type of clot formed in each case. In veins a "red thrombus" is formed, consisting of erythrocytes, leukocytes, fibrin, and platelets randomly distributed, whereas in arteries a "white thrombus" consisting mainly of platelets and fibrin strands is the obstructing lesion.[1] The predominance of platelets in this "white" clot has focused attention on their importance in arterial occlusion and has suggested that therapeutic maneuvers directed at platelet function may be more useful than standard anticoagulant therapy. This review presents the recent advances in the study of platelet morphology and function, and concludes by discussing possible therapeutic avenues.

Platelet morphology

Platelet morphology, as viewed through the electron microscope, has advanced the understanding of function (Fig. 1). This cross-sectional view of a normal human platelet shows several important structures, all of which will be discussed in greater detail later. The trilaminar membrane is covered with an amorphous "surface coat" which may be of great importance in platelet adhesion. The cell maintains, in vivo, a lenslike shape, probably because of a submembranous "skeleton," the microtubules. Cytoplasmic, or submembranous, microfibrils are sometimes seen and may participate in clot retraction. The interior of this non-nucleated cell consists of (a) mitochondria, permitting respiration and ATP production, (b) the alpha granules, which contain fibrinogen, fibrin-stabilizing factor (otherwise known as platelet factor 4), nonmetabolic ADP and ATP, and a number of enzymes,[2] and (c) the dense bodies, probably derived directly from the alpha granules,[3] which contain stored serotonin, epinephrine, and norepinephrine. A canalicular system representing tubelike invaginations of the cell membrane into the cytoplasm is sometimes visible.

Platelet aggregation and hemostasis

The first event in formation of a platelet thrombus, in vivo, takes place at the site of vessel injury when platelets begin to adhere to the damaged vessel wall and to each other.[4] Why the individual, circulating nonsticky platelet should suddenly become a communal, sessile, sticky cell is not fully understood. The intact endothelium of undamaged vessels appears to serve as a barrier that prevents platelet contact with activators in the subendothelial tissues. If endothelial disruption occurs, platelets be-

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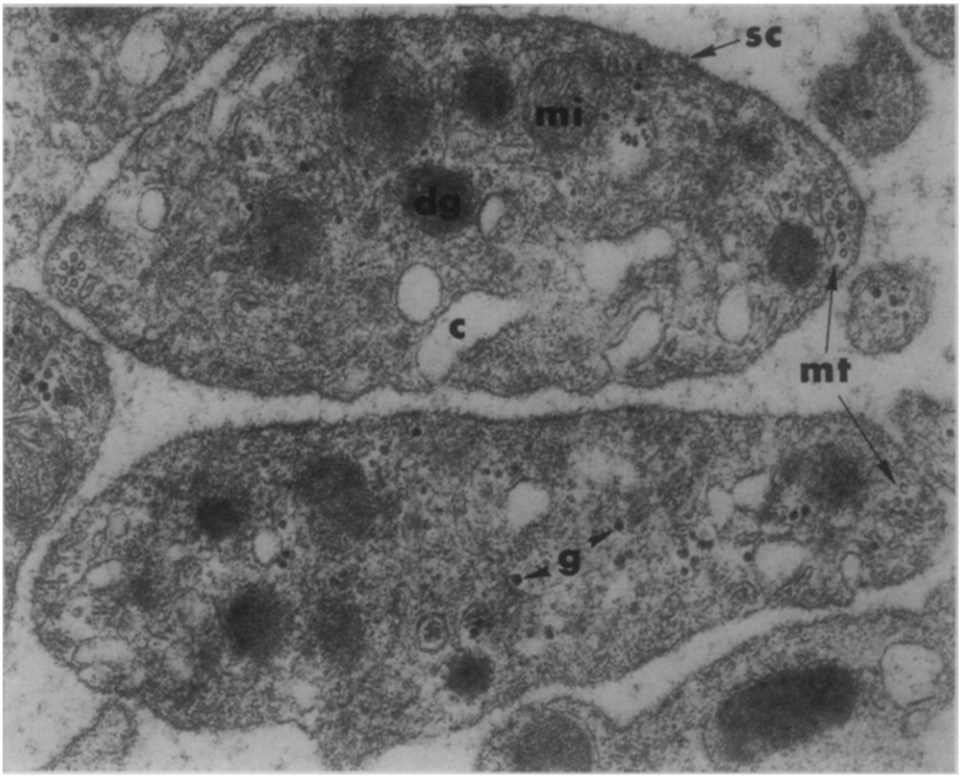

Fig. *1.* A cross-sectional view of two normal human platelets. Note the biconvex shape best seen in the upper platelet. *sc:* Surface coat and trilaminar membrane. *mi:* Mitochondria. *dg:* Dense granule. *c:* Canalicular system. *mt:* Microtubules. *g:* Glycogen granules.

gin to adhere to the injured area.[5] This may be due to exposure of collagen fibrils which are potent inducers of this phenomenon,[6] to the elaboration of ADP in endothelial cytoplasm, which if released by injury, might also activate platelets,[7] or to possible changes in the electrical surface potential of normal endothelium which repels platelets.[8] This adhesiveness cannot be prevented by heparinization.[9]

In these circumstances, platelets not only stick to injured vessel walls but also to each other. The growing platelet mass initiates hemostasis by mechanically plugging the gap in the vessel wall. This attraction of one platelet to another, called *aggregation*, is another poorly understood phenomenon. Probably of great importance here, though, is the surface coating of the platelet membrane. This material is of irregular thickness, and consists of sulfated acid mucopolysaccharides.[10] It is part of the platelet membrane, not just absorbed material, and it is present between the demarcating membranes of developing platelets in the megakaryocyte.[11] Plasma constituents, including fibrinogen, are adsorbed to this material. Indeed, fibrinogen, whether of plasma or platelet origin, calcium, and other plasma cofactors that are less well defined seem to be necessary for aggregation in vitro.[12-14] Conversely, fibrinogen breakdown products inhibit platelet aggregation.[15] Within grossly normal limits, however, fibrinogen levels in vivo do not correlate with speed or completeness of platelet aggregation.[16]

Aggregation, per se, does not result in platelet destruction or irreversible change in these cells (Fig. 2). Membranes remain intact, and a gap of 200 to 300 Å separates the aggregated cells. This gap is bridged by radially aligned fibrillary material (Fig. 3), the exact nature and significance of which are uncertain.[17] Regardless of the inciting event, platelet aggregation seems to be mediated by adenosine diphosphate (ADP).[18] Collagen, epinephrine, and thrombin are three potent aggregating agents, but each acts by causing platelets to release

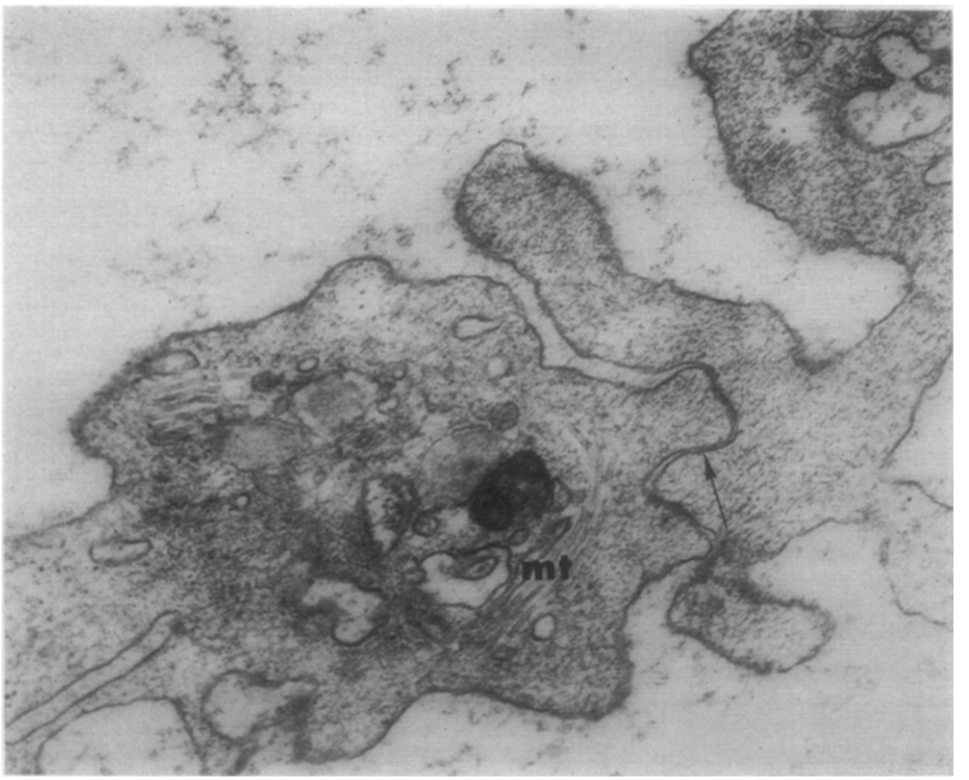

Fig. *2.* Human platelet aggregate. The microtubules (*mt*) have been sectioned longitudinally. Pseudopod formation is seen. Note the preservation of cell membrane integrity (*arrow*).

endogenous ADP stores, which, in turn, promote clumping. Endogenous ADP seems to be more potent in this regard than is exogenous ADP.[14] This effect of ADP is short-lived, and platelets will disaggregate even when appreciable amounts of ADP remain in the surrounding medium.[19]

Why ADP produces platelet aggregation is not well understood. It may alter the normally negative surface charge that all platelets carry and reduce their propensity to repel one another.[8,20] Some authors theorize that an ATPase, an enzyme which normally dephosphorylates ATP to ADP, resides in or near the platelet membrane.[21,22] This enzyme may be continually active, and through this activity may keep the platelet unsticky. ADP, however, may inhibit this ATPase and thereby cause aggregation.

Another consideration of importance is the presence on the platelet membrane of adrenergic receptor sites (Fig. 4). When catecholamines incite platelet aggregation, their effect is mediated through alpha receptors and can be inhibited by alpha blockers. Beta receptor stimulators, such as isoprenaline, cause disaggregation in vitro.[23] The beta receptor blocker, propranolol, is inactive except at high concentration. As in other tissues, catecholamines may act by influencing adenyl cyclase, the enzyme which catalyzes the formation of cyclic AMP. Epinephrine has been shown to inhibit the normal synthesis of cyclic AMP in intact platelets after prostaglandin E 1 stimulation. Phentolamine, an alpha antagonist, blocks this action of epinephrine.[24] Moreover, methyl xanthines, such as caffeine, reduce platelet aggregation by inhibiting phosphodiesterase, an enzyme which degrades cyclic AMP. In this case, therefore, aggregation may be induced by those influences which diminish platelet stores of cyclic AMP.

Platelets undergo physical changes in aggregation. Probably as an initial phenomenon representing primary interaction between some aggregating agents (ADP, collagen fibrils) and platelet membrane, the platelet changes its shape from disclike to spherical, and small spiny projections ap-

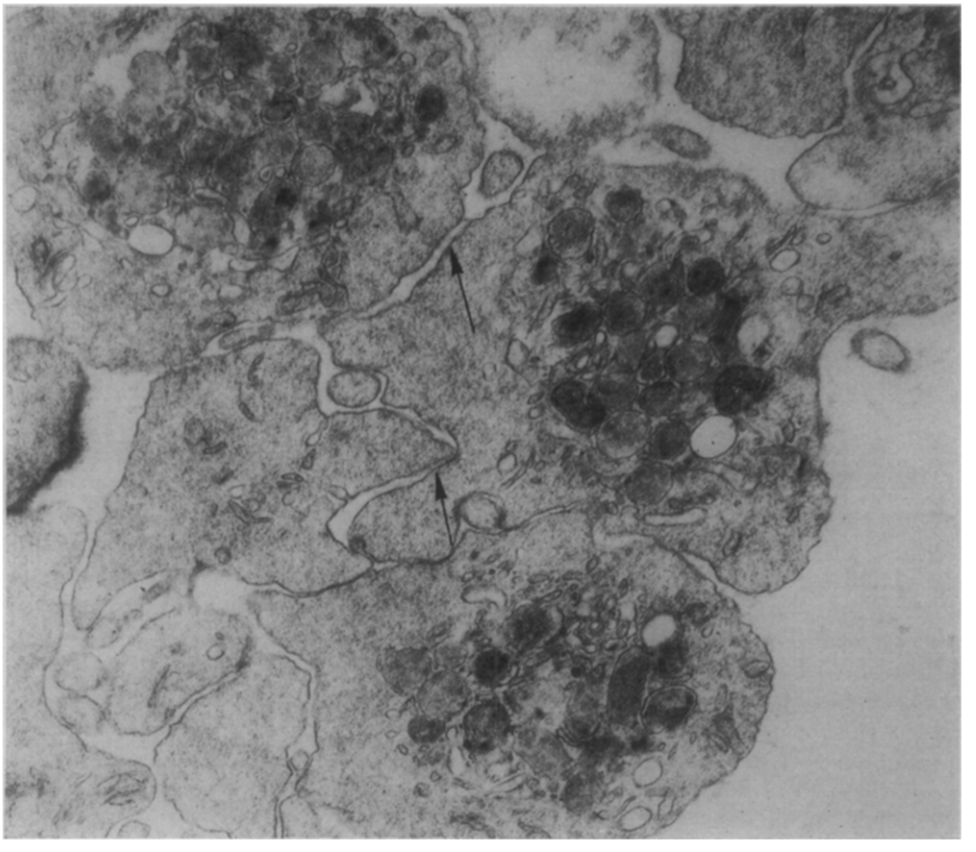

Fig. 3. Human platelet aggregate. Modest granular centralization has occurred. Fibrillary material is seen (*arrows*) spanning the interplatelet gap between intact cell membranes.

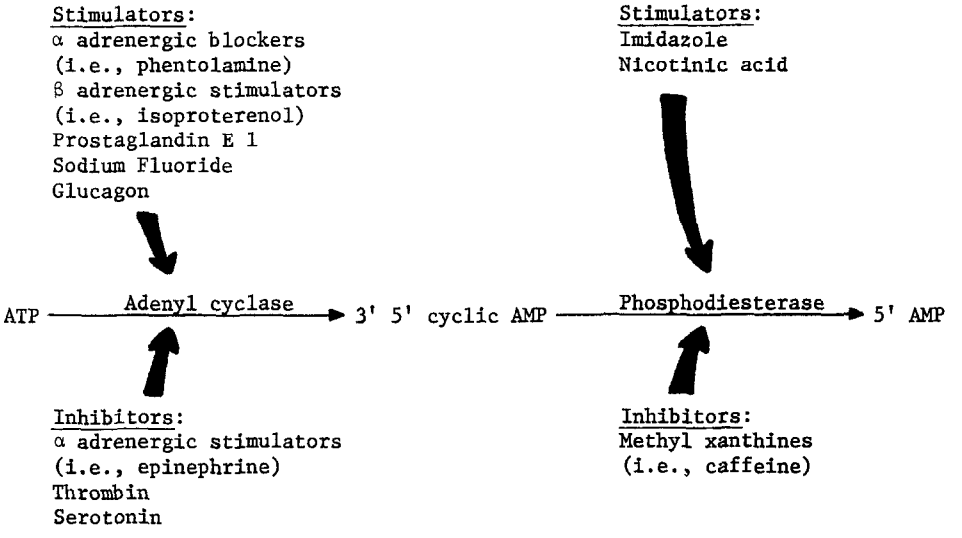

Fig. 4. Outline of 3′ 5′ cyclic AMP metabolism in human platelets. Factors promoting increased synthesis or reduced destruction of 3′ 5′ cyclic AMP inhibit platelet aggregation. The converse is also true.

pear on its membrane. In doing this it gains up to 30 per cent in volume.[21,25] Platelet aggregates induced by epinephrine, however, consist mostly of discoid forms.[26]

As mentioned previously, ADP-induced aggregation in itself produces no irreversible changes in platelet structure and function. In vivo, however, although platelet

ADP still appears to be an important mediator, other agents produce profound effects on the platelet, eventually leading to its destruction. Of physiologic importance are thrombin, produced by the action of multiple coagulation factors on prothrombin, and collagen, found in subendothelial tissue. Other inducers of this phenomenon include trypsin, papain, aggregated gamma globulin, antigen-antibody complexes, latex particles, endotoxin, and epinephrine.[27] The change in platelets which they catalyze is known as *the release reaction.*

The release reaction

The importance of the release reaction is that it converts the fragile and readily dissociable platelet aggregate into an irreversibly bound platelet plug which seals the vessel disruption. The platelets undergo visible alterations. The membrane becomes irregular, pseudopods appear, and these interdigitate with those of adjacent platelets to produce a network. In addition, the internal organization of the platelet changes drastically. The inclusions of the platelet become centrally located rather than diffused throughout the cytoplasm.[28] This granular centralization is an active process, probably mediated by the microtubules which formerly served as the platelet's "skeleton" and previously were found just beneath the membrane, encircling the equator of the cell. These now contract, perhaps in response to an influx of calcium, and appear to sweep the platelet's granules to the center of the now spherical cell.[29,30] The platelet then degranulates, releasing a number of substances into the surrounding medium. Granules are not extruded whole, but probably empty their contents into a canalicular system. The reaction begins promptly upon exposure to the inducing agent, as in the case of thrombin, where lag time is less than a second, and proceeds to completion within 40 seconds.[2]

By no means is release accomplished by a nonselective increase in membrane permeability. Instead, evidence is accumulating that platelets selectively and actively "secrete" specific fractions of stored constituents and retain other fractions.[31,32] A case in point is the release of stored adenine nucleotides. If platelets are incubated with ^{14}C-tagged adenine, they synthesize radioactive ATP and ADP. If these same platelets are then challenged with thrombin, however, despite the fact that 60 per cent of the total adenine nucleotide content is released to the surrounding medium, virtually no radioactivity is recovered there.[32,33] This strongly suggests that the cell produces and stores a nonexchangeable pool of nucleotides for just this purpose. A similar mechanism has been found in the case of platelet potassium.[2]

The substances of importance, in addition to ATP and ADP, which are discharged during the release reaction include potassium, serotonin, some enzymes, platelet factor 4, proteins, and fibrinogen. These are located principally in platelet granules. On the other hand, cell constituents found in the soluble fraction of cytoplasm, membranes, or mitochondria are retained during the reaction. These are most of the cell's enzymatic machinery, including such lysosomal enzymes as acid phosphatase, proteins, lipids, metabolically active ATP and ADP, and some of the cell's fibrinogen.[2]

The functions of these released constituents have already been alluded to. ADP serves to induce aggregation of additional platelets to the growing platelet plug, and fibrinogen is a necessary cofactor in the same process. The role of serotonin is less clear, however. This substance may not be produced by platelets, but may be absorbed from plasma through the platelet's canalicular system, and concentrated and stored in dense granules in the cytoplasm as a calcium chelate.[3,34] Patients with carcinoid syndrome show some minor morphologic abnormalities but have relatively normal platelet function.[35] Serotonin is a weak inducer of aggregation[1,18] and, other than having vasoconstrictive properties, may play no significant role in hemostasis or coagulation. Indeed, the platelet may serve only as a storage area and transporter for this amine.

Platelets and the coagulation system

At this point, unless a coagulopathy exists, blood-clotting systems are activated. Interaction between plasma and platelet coagulation factors then becomes impor-

tant. A highly simplified version of the currently accepted coagulation schema indicates two points of interaction (Fig. 5). Factor X, whether activated by the plasma intrinsic system or the tissue extrinsic system, requires the presence of factor V and a lipid for conversion to prothrombin activator, the substance necessary for formation of thrombin.[36] In the intrinsic cascade, this lipid is platelet factor 3, a lipid or lipoprotein complex whose activity seems bound to platelet membrane complexes. In vitro, such intracytoplasmic complexes are seen after the addition of thrombin to intact platelets and arise from pre-existing alpha granules.[37] This factor, which may be extruded into the surrounding environment, becomes available during the release reaction. It is also possible that the morphologic changes accompanying this event may unmask active surface lipid sites.[38] A second area of concern is the construction of a tight fibrin meshwork after its initial polymerization. This is mediated via factor XIII, also known as platelet factor 4 or fibrin-stabilizing factor. This large protein molecule is synthesized directly by platelets and is secreted into the environment during the release reaction.[39,40] It functions by catalyzing the formation of covalent bonds in the fibrin polymer, which serve as cross links to reduce the solubility and mechanical fragility of the new clot. It also inhibits the anticlotting properties of fibrin breakdown products and interferes with fibrinolysis.[41]

A brief mention might be made of another suspected coagulation function of platelets. Some investigators have reported the ability of incubated washed platelets to clot hemophiliac blood. Since this blood is deficient in either factor VIII or factor IX, the theory is that these "activated" platelets have initiated clotting via the extrinsic system, and have resembled tissue factor in their action.[1,42] This presumed function is not universally accepted, however, and the propensity of platelets to absorb various clotting factors to their surfaces hints that the missing factors in the intrinsic system might have been added this way.[38]

Thrombosthenin and clot retraction

The growing thrombus that we have been following now needs only one more bit of remodeling by platelets before it is completed. This last but very important step is known as *clot retraction*. This reaction takes place through the active function of the platelet's contractile protein, thrombosthenin.[43] As noted previously, the formation of pseudopods is a morphologic change accompanying the release reaction. These projections contain a number of microfilaments which lie beneath the cell membrane and are dispersed throughout the cytoplasm. These microfilaments are synthesized in the megakaryocyte, and, although they strongly resemble the subunits of human striated or smooth muscle, fluorescent antibody studies have not shown any cross reactivity.[44] The contractile substance itself appears to be a highly asymmetric protein, of high molecular weight, which exists in fibrillar form, and which, in some studies, appears to have a periodic structure like that of actomyosin.[30,45-47] Studies of thrombocytes in fishes have shown that the protein may be stored in vesicles in the resting cell and is polymerized into fibrillar form only during activation.[48] Electron micrographs of microtubules, the submembranous structures discussed earlier for their role in preserving cell shape, show 13 to 15 subunits, which again strongly resemble microfibrils.[30] It would appear, therefore, that microfibrils and microtubules of platelets represent two morphologic forms of the same contractile protein.[49]

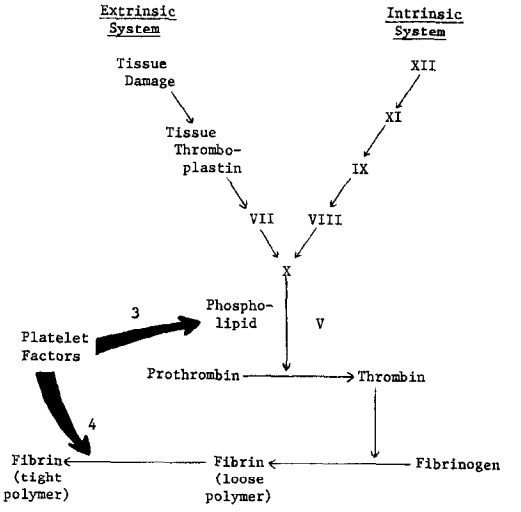

Fig. 5. Schematic diagram of the coagulation cascade. The heavy arrows point to known sites of action of platelet factors.

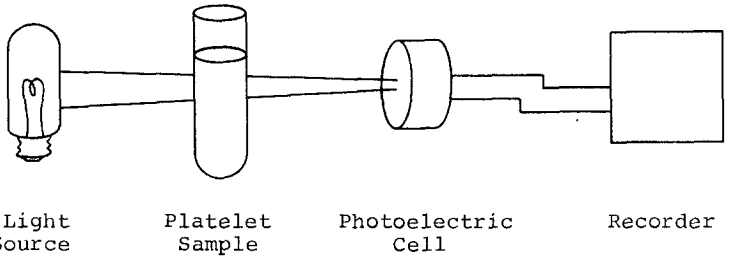

Fig. 6. Schematic of device to measure platelet aggregation.

The stimulus for contraction is unknown, but the process appears dependent on ATP and calcium. Indeed, thrombosthenin itself embodies an ATPase activity which may control some of the metabolic steps leading to aggregation and release as well as clot retraction.[22,31,44,66] The contraction, therefore, of thrombosthenin in the pseudopods of activated platelets consolidates the clot and wrings serum from it, producing a completed thrombus. The platelet, its hemostatic and coagulation functions completed, now dies and is disrupted. It is generally removed from the thrombus as healing progresses, although platelet antigen is present for as long as 6 months in the fibrous plaques resulting from experimental mural thrombosis in animals.[50]

Other platelet functions

As if all these complex functions of this remarkable bit of cytoplasm did not suffice, other capabilities have been ascribed to it. Platelets seem able to phagocytize virus and other particulate material, such as Thorotrast.[43] Carbon particles injected intravenously into rabbits are probably transported to the reticuloendothelial system by platelet aggregates.[51] The significance of these properties in man's immunological defenses awaits further study. Furthermore, platelets aggregate and release when incubated with antigen-antibody complexes, and, in so doing, secrete a factor (or factors) which leads (lead) to increased permeability of the vessel walls.[52] This may be mediated by a cationic protein found in extruded platelet granules which stimulates mast cells to liberate histamine. The latter, in turn, may produce the observed increase in vessel permeability.[53] The positivity of the Rumpel-Leede test in thrombocytopenia may relate to a third ancillary platelet function. If radioactively labeled platelets are transfused into thrombocytopenic guinea pigs, the endothelial cells of the blood vessels are seen to bind and incorporate platelet cytoplasm into themselves rapidly.[54,55] This suggests that platelets may play a primary metabolic role in the nourishment and maintenance of intact vascular endothelium.

Tests of platelet function

One of the principal difficulties in understanding platelet function is that of measuring it. The platelet count is only a quantitative measurement, and clot retraction, although roughly evaluating thrombosthenin content, is still dependent on and proportional to the platelet count.[43] Platelet factor 3 activity may be estimated by its accelerating influence on the clotting effect of viper venom, but this study has inadequacies and is not widely available.[1] Platelet factor 4 or fibrin-stabilizing-factor activity can be approximated by studies of clot solubility in urea and monochloracetic acid and of clot elasticity, but again these are not widely available.[41] Two studies which are becoming widely accessible for platelet evaluation are the glass-bead-column procedure for estimating "adhesiveness" and the photometric measurement of platelet aggregation.

The first study is simple in design. A platelet count is done on citrated whole blood, which is then passed, at a constant velocity, through a known volume of glass beads of uniform size. A second platelet count is done on the blood emerging from the column. The difference between these two counts, or that percentage of platelets which remains in the column, is taken as a measure of platelet "adhesiveness."[56] This study is reproducible in experienced hands,[57] but its clinical usefulness in predicting the risk of thrombotic events in

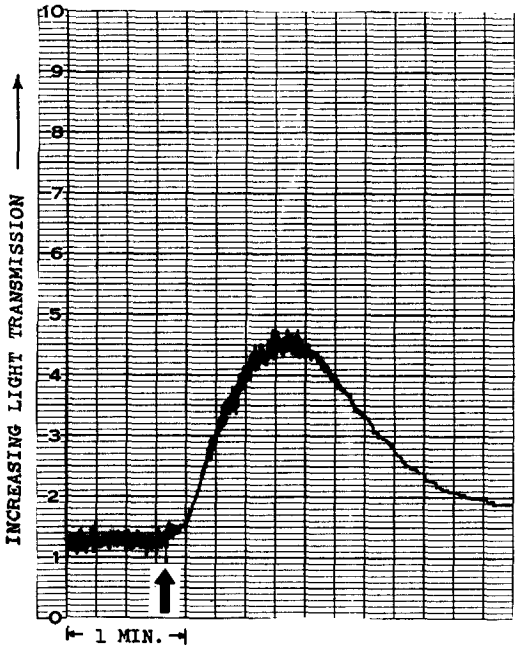

Fig. 7. Platelet aggregometer record. Aggregating agent used was ADP (*added at arrow*).

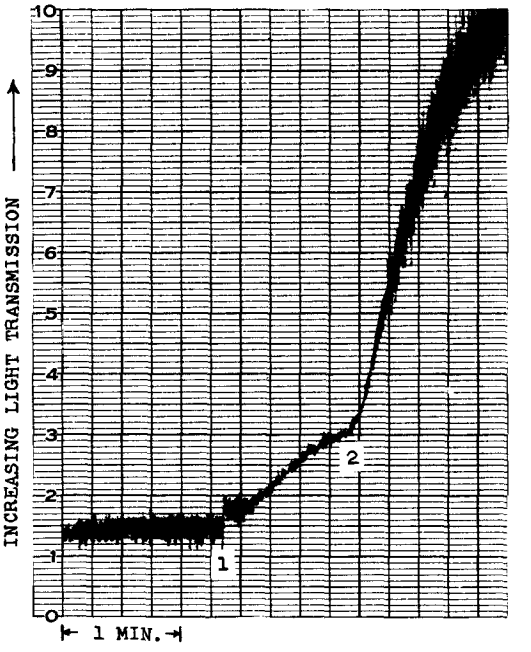

Fig. *8*. Platelet aggregometer record. Aggregation induced by epinephrine (added at *1*). Note two-phased response produced by late release of platelet ADP stores (*2*).

anticoagulated patients has been questioned.[58]

The second study is more complex (Fig. 6). A beam of light of constant intensity is focused on a photoelectric cell which constantly samples the light transmitted and records this on a slowly moving papergraph. Platelet-rich plasma, which is constantly stirred, is placed in this chamber in the path of the beam. Baseline transmission of light through the platelet mixture is obtained, and then an aggregating agent of choice is added. This induces platelet clumping, which tends to clear the solution and increase the transmission of light through it. A typical record from such a study is shown in Fig. 7. The aggregating agent used was ADP. Time is noted on the abscissa, and increasing transmission of light, on the ordinate. After the addition of ADP to the system, a brief time lag is noted, and then aggregation proceeds. After it reaches its maximum, there is a slow disaggregation phase represented by this slowly decreasing transmission of light. Several measurements may be made here, including the slope of this line, representing the speed of aggregation, the maximum amount of aggregation achieved, and the time needed for 25 per cent disaggregation.

Fig. 8 shows an aggregation curve made with the addition of epinephrine. A two-phased response is seen, the first phase caused by epinephrine's own effect on platelets, and a second more profound rise as platelet stores of ADP are released in response to the initial stimulus. Retention of platelets in the glass-bead column is directly proportional to the initial rate of platelet aggregation induced by ADP or thrombin, thereby relating these two studies and suggesting that aggregate formation in the column may be the mechanism of platelet sequestration there.[59]

Factors modifying platelet function

Many metabolic situations and therapeutic agents alter these measurements, and thereby give some indication of their effect on platelet function. For example, surgery itself usually produces not only an increase in platelet count, but a similar rise in the rate and extent of aggregation when measured from about the tenth to the twentieth postoperative day.[60,61] Diabetic and normal volunteers rendered hyperglycemic by intravenous infusions of glucose were found to have increased platelet

"adhesiveness."[62] One group found that the feeding of high cholesterol diets to rabbits led to beta lipoprotein changes which increased platelet "adhesiveness" and aggregation rate,[63] but these findings were not confirmed by another group which fed an atherogenic diet to rabbits.[64] In one study, patients with recent acute myocardial infarctions, thrombophlebitis, or major arterial occlusions have demonstrated increased platelet "adhesiveness" when compared to controls,[65] but others have not demonstrated increased platelet aggregation in the immediate postmyocardial infarction period.[66] The direct applicability of such work to human vascular disease and the need for further study are evident.

In patients with Waldenström's macroglobulinemia, platelet aggregates form poorly[67] and platelet factor 3 activity develops subnormally. This may be related to effects of the abnormal serum protein on platelet membranes, and can be partially reversed by incubation of the patient's platelets with antimacroglobulin antibody.[68] Although pheochromocytoma is not associated with thrombotic complications, reduction in platelet "adhesiveness" has been demonstrated after removal of the tumor.[69]

Drugs also alter platelet function. As alluded to previously, phentolamine, an alpha receptor blocker, inhibits the aggregation of platelets by epinephrine.[18] Propranolol and nitroglycerine inhibit both ADP and epinephrine-induced aggregation in vitro, but have little effect in vivo.[70] Clofibrate, despite some initially promising reports, probably does not have an important effect on platelet function.[71] Phenothiazine derivatives seem to interfere with release of platelet stores of ADP, perhaps through their membrane-stabilizing effects.[1] Aspirin and, for that matter, sodium salicylate, indomethacin, mefenamic acid, and phenylbutazone appear to have a similar membrane-stabilizing effect, and prevent release of platelet constituents after appropriate induction.[72-74] Aspirin has a particularly prolonged effect, lasting up to 7 days after a single dose, and exerts a particularly strong action on preventing the appearance of platelet factor 4 activity.[75] It may act by acetylating active sites on platelet membranes, and intracytoplasmic constituents.[76]

In vitro, heparin in large dosages decreases aggregation perhaps because it is a strong organic acid.[20] Coumadin, on the other hand, actually increased the extent of aggregation in anticoagulated patients serving as their own controls.[77] Colchicine, when added to platelet-rich plasma, inhibits platelet aggregation, reduces "adhesiveness," and diminishes clot retraction, the latter probably by interference with microtubular function.[78]

Two naturally occurring compounds deserve comment, inasmuch as each is a strong inhibitor of platelet aggregation in vitro and in vivo. The first is prostaglandin E 1, which acts through stimulation of adenyl cyclase and increases in platelet cyclic AMP stores.[79-81] The second is adenosine, which results from the metabolic degradation of ATP and ADP. Its effect, however, is transient because of rapid deamination in tissues and blood, and its hypotensive action is significant.[82]

Dipyridamole, an antiplatelet drug

The discovery that dipyridamole inhibited the deamination of adenosine by intact human red blood cells suggested the usefulness of this drug as a platelet inhibitor.[83] The platelet-inhibiting effects of dipyridamole became more prominent after publication of a well-controlled study, in 1968, showing statistically significant reduction in arterial embolic events in a treated group of patients with prosthetic heart valves.[84] Subsequent enlargement of this study has shown increasingly significant protection for the dipyridamole-treated patients.[85] More recently, platelet survival has been found uniformly shortened in anticoagulated patients with artificial valves, and platelet lifespan can be extended to normal by adding dipyridamole to the treatment program.[86]

The direct effects of this drug and its analogues on platelet function in vitro are a significant decrease in "adhesiveness," as measured by the glass-bead technique, and a depression of aggregation, with enhancement of disaggregation noted in some studies.[87-89] Another study showed no effect on disaggregation, however.[90] In vivo animal experiments have shown the effectiveness of this drug in preventing thrombus formation in intentionally damaged arteries.[89,91]

Measurable changes in platelets of patients taking dipyridamole chronically were harder to demonstrate. Although one group found a significant decrease in platelet "adhesiveness" in 4 of 6 treated patients with coronary artery disease,[92] other authors found no change in similar patients.[86-88,93]

Work from our laboratory also showed the difficulty of demonstrating the in vivo effect of dipyridamole on platelet function.[94] Four warfarin-anticoagulated recipients of prosthetic heart valves were studied. After baseline platelet function* and standard coagulation determinations,† dipyridamole was administered (100 mg. orally four times a day) for a period of 7 to 30 days. The studies were then repeated, the drug was discontinued, and studies were obtained again 2 to 4 weeks later. The results did reveal a significant reduction in clot retraction during administration of dipyridamole, but no other statistically significant difference in platelet function or coagulation parameters during the control and treatment periods.

Dipyridamole is partially bound to red cell membranes, and may thus prevent adenosine from diffusing into the cell, where it is normally metabolized.[82] It also appears to inhibit adenosine deaminase, the responsible enzyme, directly.[83] The drug may render platelet membranes impermeable to adenosine as well.[95] Although most authorities believe that the resultant extracellular accumulation of adenosine then disturbs platelet function, some think that a direct effect of dipyridamole on the platelet membrane may be responsible.

The purpose of this discussion is to emphasize the primary role of platelets in the genesis of arterial thrombosis and the fact that antiplatelet agents, such as dipyridamole, do exist and may be useful, alone or in combination with standard anticoagulants, in preventing arterial occlusion or embolization. The usefuluess of such drugs may extend beyond common forms of arterial disease. Recent reports of platelet aggregates in renal arterioles and glomeruli of patients with lipoid nephrosis, glomerulonephritis, and transplanted kidneys undergoing chronic or hyperacute rejection reactions point to another possible use of these agents.[97-100] Although dipyridamole by itself does not prevent severe rejection reactions,[101] it has been shown, when used with Coumadin-like drugs or heparin, or both, to give significant protection against intrarenal vascular occlusion in such circumstances.[102] Its record of safety and low incidence of important side effects should make it an attractive antiplatelet agent for further study.

*Studies included ADP, epinephrine, collagen-fibril–induced aggregation, and whole blood platelet "adhesiveness."

†Studies included platelet count, clot retraction, one-stage prothrombin time, partial thromboplastin time, thrombin clotting time, and euglobulin lysis.

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