A Method for Evaluating the Platelet Surface Response by Using Electron Microscopy

Jeanne M. Riddle
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Our method for evaluating the surface response of any given platelet population, which has evolved over the past two decades, provides a measure of both the adhesive and cohesive qualities of platelets. General morphologic features of the adherent, whole platelets can be assessed by transmission electron microscopy, and the results are reproducible when serial samples are collected on normal subjects and patient groups. With this technique, different metabolites and drugs can be combined with platelet populations that are either normoresponsive or hyperactive to determine if these substances trigger or inhibit the platelet response. To date, the results from in vitro studies have paralleled in vivo findings during clinical trials. The method appears suitable for assessing the degree of surface response for any platelet population, determining the in vitro action of a variety of substances on the platelet response, and for monitoring the antiplatelet capacity of established as well as experimental therapeutic agents.

Introduction

Early investigators demonstrated that platelets removed from the interior of blood vessels and placed in an anticoagulated, in vitro system exhibited structural alterations. If the anticoagulated sample of blood was exposed to a foreign surface such as the exterior of a glass microscope slide or support films used in electron microscopy (collodion and Formvar), an ordered sequence of shape changes took place (1-3). Rebuck’s appreciation of the significance of these structural changes led us in 1958 to devise a method to assess the surface response of a platelet population in normal subjects and disease states. We chose the transmission electron microscope over the light microscope because of its greater resolving power and increased range of magnification.

Materials and Methods

Our original method was first published in 1959 (4)** and modified a technique previously used by Braunsteiner (3,5). We were able to identify four classical stages of surface activation during a platelet differential count that evaluated surface response for 100 successive platelets. These four were: round, dendritic, intermediate, and spread types (6). Since 1958, we have revised our method by introducing commercially available, nonwettable equipment, by changing the anticoagulant from heparin to 3.8% sodium citrate, and by combining the intermediate and spread types in order to simplify the differential count (7). Our current method is as follows:

Venipuncture procedure

Peripheral blood is obtained by venipuncture (Fig. 1) using a siliconized Butterfly infusion set with an attached 21 gauge needle (Abbott Hospitals, Inc, North Chicago, Ill). Individual samples are collected into sterile, disposable plastic syringes (Becton, Dickinson, and Co, Rutherford, NJ). First, a 2 ml whole blood sample is withdrawn and discarded to eliminate any procoagulant substances that might have been introduced during venipuncture. A second syringe containing 1 ml of 3.8% sodium citrate is attached immediately to the tubing, and 9 ml of whole blood is withdrawn into the anticoagulant, which is mixed briefly with the whole blood. Additional samples can be obtained if the effect of a specific metabolite or drug is to be evaluated.

Ex vivo method of platelet collection

The anticoagulated blood specimen is transferred immediately to a 6 oz polyethylene, square bottle with a wide mouth (Nagle Co, Rochester, NY), and a nonfrosted micro-
scope slide covered with a thin film of Formvar (1% Formvar dissolved in ethylene dichloride) is introduced horizontally (Fig. 2). The system either remains at room temperature or is placed in a 37°C incubator. A stopwatch is started, and the blood mixture remains in contact with the Formvar film for exactly eight minutes. During incubation, blood platelets and other activated cellular elements attach to the surface of the Formvar film. Afterwards, the microscope slide is removed from the bottle, drained briefly, and rinsed in Tyrode’s solution previously warmed to room temperature. Rinsing continues until erythrocytes are no longer grossly visible on the film surface. The slide is immersed in either cacodylate buffered 3% glutaraldehyde (8) or 1% buffered osmium fixative (9) for 15 minutes at room temperature to stabilize the structure of the adherent cellular elements. After fixation, the slide is rinsed in several changes of distilled water to remove the buffer salts. The preparation is then air dried (Fig. 2).

Next, the edges of the slide are scraped with a No. 22 Bard-Parker blade to free the Formvar film. The platelet population adhering to the surface is surveyed with a phase microscope, and stainless steel, 200 mesh specimen grids (Ernest F. Fullam, Schenectady, NY) are placed over representative areas so that their convex surfaces contact the Formvar film. The final mounting of the preparation is outlined in Fig. 3. After mounting, platelet populations are viewed with a transmission electron microscope operated at 50 KV.

**Platelet types**

Based on their degree of surface activation, three distinct types of platelets are observed in these preparations. The round type is compact, has a smooth contour, and is uniformly electron-dense. The dendritic type has a compact, electron-dense central area and several extruding pseudopodia (7). The spread type contains transitional forms that exhibit varying amounts of hyalomeric fusion and spreading as well as obvious centralization of their cytoplasmic organelles (27). Platelet aggregates are observed also. Sometimes these are composed of a single platelet type; sometimes they contain an admixture of types and exhibit peripheral cytoplasmic spreading.
Platelet differential count

Our differential platelet count of 100 successive platelet types measures the capacity of platelets to: 1) adhere to a foreign surface; and 2) cohere and form aggregates. Values for round and dendritic types, indicative of minimal surface activation, are combined, and a single value for spread types, including the transitional intermediate type, is recorded. Round and dendritic platelets consistently dominate the differential counts of asymptomatic subjects, with a mean value of 88% ± 7%. On the average, the spread type comprises only 12% ± 7% of the differential count in the control group. The number of platelet aggregates per differential count is noted also. The aggregating tendency of platelet populations from the control group varies over a wider range, with an average of 68 ± 50 aggregates per 100 single platelets counted.

The reproducibility of our method was established by performing serial differential counts on platelet samples from several subjects at various time intervals. The consistency of our data demonstrate that our method can be applied to assess the activation quality and aggregating capacity of platelet populations from a single subject during follow-up studies in clinical trials.

Modifications for scanning electron microscopy (SEM)

A recent modification of our standard method uses the scanning electron microscope (7, 10-12) to display the surface topography of adherent single platelets and aggregates.

Platelets are collected onto the surface of the Formvar film, which is rinsed with Tyrode's solution as described previously. Fixation is accomplished by exposing the adherent platelet population to cacodylate buffered 3% glutaraldehyde for 1 hour at room temperature. At this point, two routes of final processing are possible. In one, the preparation is rinsed in distilled water and the exterior surface of the platelets is stabilized by a brief exposure to Parducz's fixative (13) for 15 minutes at room temperature. The doubly fixed sample is then rinsed once again with distilled water and placed in 70% ethanol for 5 minutes. Next, the preparation is withdrawn and allowed to air dry. If air drying is not desirable, the platelets fixed with glutaraldehyde can be stabilized by critical point drying. In the final step before SEM viewing, a thin layer of gold-palladium is deposited over the exterior of the preparation by the sputtering technique.

Applications of the Method

Clinical studies of selected disease states

By obtaining a pictorial TEM record of the activation state of certain populations of platelets in several diseases, we have been able to pinpoint the origin of functional platelet faults and to establish a relationship between the observed platelet response and the basic etiologic mechanism of these disease processes.

When platelet populations from patients with untreated pernicious anemia were studied, the dendritic type of platelet had defective pseudopodia. They were few in number, short, and had a blunted end (Fig. 4). Even after appropriate treatment, this defect persisted, just as some erythrocytes also do not revert to an entirely normal state (14).

An altered ability to form pseudopodia was also detected in two other diseases, idiopathic thrombocytopenic purpura (ITP) and Waldenstrom's macroglobulinemia. In ITP, the pseudopodial processes were abortive and malformed so that they appeared as short, rather broad extensions with a flattened end. The border of some of these platelets therefore presented a serrated, gear-like appearance (15). It was suggested that the antiplatelet antibodies present had attached to small, distinct plateaus at affected sites. (Fig. 4). These findings were similar to those produced when known antierthrocytic antibodies were exposed to the surface of sensitized erythrocytes. In Waldenstrom's
macroglobulinemia, pseudopodial formation appeared to be prevented (16) because the entire platelet surface was coated nonspecifically with the macroglobulin (Fig. 4). In view of more recent advances, it may be that this coating prevented triggering substances from reaching specific receptor sites on the platelet surface.

A direction for determining the functional abnormality associated with thrombocytopenia A was suggested when platelet differential counts from these subjects consistently exhibited a distinctive spread type with a well-defined, electron-dense central zone (Fig. 4). This finding implied that essential substances were not being released from the platelet during its surface activation sequence. When these platelets were subjected to sonic oscillation or extracted with distilled water, the hypothesis was substantiated because platelet factor 3 was released in normal amounts, and the altered prothrombin consumption, a regular feature of this disease state, was corrected (17,18).

In more recent years, our modified method has been used to help determine the prethrombotic state and to evaluate an ongoing thrombotic episode. When we evaluated the platelet populations of patients with clinical myocardial infarction during late stage convalescence (7,19), not only was there increased surface response coincident with the infarction, but a hyperactive platelet response persisted in 67% of these patients. These data suggest that if both increased surface activation and increased aggregation continue, the patient may be predisposed to future thrombotic episodes despite apparently satisfactory clinical recovery from the initial myocardial infarction.

In another series of studies, the platelet response of patients with varying degrees of cerebrovascular disease was determined. During an acute stroke phase, platelet populations from 85% of the 31 patients tested were hyperreactive, while increased platelet activity was noted in only 36% of 25 patients with chronic cerebrovascular disease (10, 20-22). Of 20 patients with an established diagnosis of transient ischemic attacks, only 30% had platelet populations with increased surface activation (23).

Certain forms of arthritis, e.g., primary gout (24) and rheumatoid arthritis, also have clinically evident thrombotic episodes as a part of the total disease process. To date, we have evaluated platelet populations from 124 patients with primary gout. More than half (55%) demonstrate primed platelet populations so that their degree of surface activation and/or tendency to form aggregates is abnormally increased (25). Our studies suggest that the episodes of arterial thrombosis in gout may be related to the platelet hyperactivity induced in these patients by the associated hyperuricemia and possible deposition of urate crystals within vessels.

We have also determined platelet reactivity in patients with classical rheumatoid arthritis. Increased platelet surface activation was found in 8 of 25 women and 8 of 15 men or 40% of these patients (26). Although the level of platelet response did not correlate with either the presence of rheumatoid nodules or the titer of rheumatoid factor, it did correspond with serum urate level above 5 mg%. In rheumatoid arthritis, platelets may also be activated by interactions between platelets and immune complexes, since these antigen-antibody combinations are known to trigger the platelet surface response.

**Metabolic studies**

Our basic method can be adapted to monitor alterations of the platelet response by any substance that is water soluble. Comparison of the results obtained from the differential counts of the collected platelets in the absence and presence of such a substance demonstrates whether it exerts a direct potentiating action or an inhibitory effect on the surface response of the platelet population chosen for study.

To determine whether or not a metabolic product that increases in response to a disease process will trigger surface activation, platelet populations from asymptomatic subjects should be used. On the other hand, to determine the inhibitory capacity of drugs that might aid in treating clinical disease states involving thrombosis, platelet populations from patients with an abnormally activated surface response should be used.

As an example of the former, we combined varying concentrations of uric acid with normal platelet populations from six asymptomatic subjects to determine if the hyperuricemia found in primary gout might serve as a triggering mechanism for the increased platelet response associated with over half of the gouty patients. As we increased the concentration of urate, we observed a corresponding elevation in the percentage of spread types and platelet aggregates (27). At levels of urate comparable to those seen in primary gout patients, the platelet differential count of the test system mimicked that found in the primary gout patients who demonstrated an increased level of platelet reactivity.

Since blood levels of fibrinogen split products (FSP) and biologic amines increase during minor trauma and in acute thromboembolic disease, the effect of FSP (28-30), epinephrine, norepinephrine, and serotonin on the surface response of platelet populations from asymptomatic subjects was evaluated (11). Serotonin and FSP significantly increased their capacity for cohesion so that increased numbers of platelet aggregates were present, while epinephrine, in addition to increasing aggregation, also accel-
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derated hyalomeric spreading. The extent of the altered platelet response was concentration dependent. These studies provide indirect evidence that as the stressful process of thrombosis occurs, release of these biogenic amines and products of fibrinolysis may prime the circulating blood platelets so that they contribute further to the disease process.

Studies with pharmaceutical agents

We have added a variety of therapeutic agents to platelet populations from patients with primary gout to evaluate their potential inhibitory action on an abnormally increased surface response. One of the concentrations represented a feasible blood level value for the drug as it is administered in an ordinary clinical setting. Using platelet populations from either three or six patients with primary gout, we found that the degree of surface response of each population was abnormally increased, that is, the percentage of spread types was consistently elevated and the amount of aggregation was generally elevated (Figs. 5, 7). Colchicine, phenylbutazone, and probenecid (Table I) did not appear to block either the degree of hyalomeric spreading or the amount of aggregation appreciably (25). We therefore considered these compounds to be noninhibitory. Conversely, allopurinol (Fig. 6), oxypurinol, sulfipyrazone, (anturan), halofenate, indomethacin (27), and periaictin were effective to varying degrees in either directly blocking the amount of hyalomeric spreading, in reducing the aggregability of the platelets, or in mediating both (Table II). Consequently, we feel that this type of study is a valuable guide in selecting drugs to be tested as potential antithrombotic agents for subsequent clinical trials.

TABLE I

<table>
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<tr>
<th>Drug Conc. (ug/ml)</th>
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<th>%S</th>
<th>P.AGG.</th>
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%R+D = percentage of round plus dendritic types counted
%S = percentage of the spread type counted
P.AGG. = platelet aggregates per 100 single platelets counted
Normal range = R+D = 88% ± 7; S=12% ± 7; P.AGG. = 68 ± 50

* = potentially attainable blood level during in vivo administration

Fig. 5
Platelet population from a patient with primary gout showing an increased number of spread type platelets and hyperaggregability (X4,700).

Fig. 6
In the presence of allopurinol, the platelet surface response of triggered platelets was inhibited in vitro so that dendritic type platelets and aggregates predominated rather than spread types (X4,500).

Fig. 7
Platelet population from a patient with primary gout before treatment. Note dominant spread type platelet and large, numerous aggregates (X4,500).
TABLE II

<table>
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<tr>
<th>Inhibitory Agents</th>
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%R+D = percentage of round plus dendritic types counted
%S = percentage of the spread type counted
P.AGG. = platelet aggregates per 100 single platelets counted
Normal range = %R+D = 88%±7; %S = 12%±7; P.AGG. = 68±50
* = potentially attainable blood level during in vivo administration

Clinical trials

Finally, we studied serial changes in the platelet surface response when a therapeutic agent is administered to patients with a defined disease process. We have monitored the activation for platelet populations before and during the daily administration of allopurinol (300-600 mgs) in 11 patients with primary gout. Platelet differential counts were performed in six before treatment and according to the following post-treatment schedule: 24 hours, 48 hours, 1, 2, 4, 6, 8, 12, 16, 20, and 24 weeks. The sampling schedule for the other five patients was less structured, and the period of treatment varied from as short as 1 week to as long as 8 weeks.

Surface activation was increased to abnormal limits with a predominance of the spread type and/or an increased number of aggregates (Fig. 7) in each of these 11 patients before treatment with allopurinol. Initially, in 9 of 11, the serum uric acid level was greater than 5.5 mg% (82%). When platelet populations from 8 patients were surveyed 24 hours after treatment with allopurinol, platelet reactivity was reduced to within normal limits in 3 of the 8 (38%). In all cases, the serum uric acid remained abnormally increased. If the results for the entire treatment series were averaged, the state of platelet reactivity was reduced to within normal limits, as demonstrated by the dominance of dendritic types (Fig. 8) for 8 of 11 (73%) patients. The serum uric acid level was reduced to within normal limits in only 4 of these 11 (36%). We observed the coexistence of a normally reactive platelet population and an increased serum uric acid level in 6 of the 11 patients (55%). These data suggest that allopurinol exerts a rather immediate in vivo inhibitory action on platelet surface activation (Fig. 9) which parallels its in vitro antiplatelet action.

Discussion

Through the years, investigations on the structure, biochemistry, and clinical importance of blood platelets have steadily increased. A variety of methods have been devised to determine the adhesive and cohesive qualities of platelet populations from both asymptomatic subjects and patient groups representing various disease states. The most popular method to monitor platelet function in today’s clinical setting is the photometric test, or so-called platelet aggregometry (31, 32). Essentially, this method records photometrically the aggregation of platelets after a triggering substance has been added to an isolated and selectively concentrated population. Its advantages are that the platelet response to a wide variety of agents can be obtained at different concentration levels and the character of the response (reversible vs irreversible aggregation) can be visualized. The disadvantages of aggregometry are that it is
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Abnormal pretreatment parameters of primary gout (hyperuricemia and hyperresponsive platelets) normalized by allopurinol therapy.

time consuming, and the platelet population being studied undergoes extensive handling, such as centrifugation and adjustment of the count per mm$^3$, before its functional capacity is evaluated.

Both the technique used by Breddin (33) and our procedure (7) provide a more direct record of platelet populations as they participate in surface activation and aggregation. The advantages of our technique are: 1) platelet populations are subjected to only minimal manipulation after blood is withdrawn; 2) steps in the procedure are simple and can be accomplished in a short time; 3) the activating surface, a Formvar film, has a uniform composition and reproducible characteristics; and 4) a pictorial record of surface activation and aggregation is available so that structural characteristics of the platelet population can be investigated. The major disadvantages of this technique are: 1) a transmission electron microscope is required to perform the platelet differential count adequately; and 2) the population of platelets evaluated may not be wholly representative of the total platelet population, if for some reason a particular functional state is selectively collected on the surface of the Formvar film during incubation.

The versatility of our technique is broad. Because results are consistent, the platelet response of a normal subject, patients with various disease states, or of different therapeutic regimens can be evaluated over a long time through serial sampling. In addition, the capacity of a substance to either trigger or inhibit a platelet's surface response can be detected, depending upon whether a normally reactive platelet population or one with increased reactivity is used to monitor the effect.

Any method, either established or prospective, that is designed to evaluate platelet response should answer the following questions: 1) Can this test system reliably select persons within the general population who are at a high risk for thrombosis? 2) Is it useful in predicting if an established thrombus will extend? 3) Does a defined biochemical alteration constitute a risk factor for thrombosis? 4) What drugs are effective antiplatelet agents both in vitro and in vivo?

Our method appears to be suitable for assessing the degree of surface response for any platelet population, for determining the in vitro action of a variety of substances on the platelet response, and for measuring the antiplatelet capacity of established as well as experimental therapeutic agents.
References