# PLATELET-DEPENDENT GENERATION OF CHEMOTACTIC ACTIVITY IN SERUM*

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The recognition that different host defense mechanisms against injury are closely interrelated has permitted a clearer understanding of the processes of inflammation, blood coagulation, and activation of serum proteolytic enzyme systems. Human blood platelets are cells primarily specialized for hemostasis. However, they have also been found to possess inflammatory functions characteristic of polymorphonuclear leukocytes and may therefore play a broader role in host defenses (1).

Leukocytes promote inflammatory responses by releasing mediators of vascular permeability and mast cell degranulation, bactericidal proteins, chemotactic factors, and lysosomal enzymes that cause tissue damage (2). Platelets also release mediators of vascular permeability (3) and bactericidal proteins (4) and possess enzymes that damage elastic tissue and collagen (5, 6).

This study has examined the role of platelets in the production of chemotactic activity for leukocytes and presents evidence that activation of the complement system by platelets is a physiologic process that forms a link between the hemostatic process and inflammation.

## Materials and Methods

*Preparation of Platelets and Platelet Fractions.*—Human platelet concentrates were prepared from whole blood anticoagulated with acid citrate dextrose and kindly supplied by the New York Blood Center and the Memorial Hospital (New York) donor room. Platelets were separated and extensively washed by methods previously described in detail (3). Contamination by leukocytes was less than 1 per $10^4$ platelets. The isolated platelets were briefly sonicated using a Branson Sonifier (Branson Instruments Co., Stanford, Conn.) and were extracted once with ice-cold 0.2 N $H_2SO_4$ for 1 h. Acid extracts were cleared by centrifugation at 12,000 $g$ and were dialyzed against buffered saline for 4 h at 10°C before experimental use. Dialyzed material was filtered through 0.45-$\mu$m pore filters (Millipore Corp., Bedford, Mass.) and used immediately or stored at −20°C for up to 2 wk.

Isolated platelet granules were obtained by sucrose density gradient centrifugation of

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homogenized, washed platelets (7). Both saline lysates and acid extracts were prepared from the granules. Protein content of preparations was measured by a modified Folin method (8).

Washed fresh platelets for aggregation studies were prepared by the method of Ardlie (9) omitting apyrase from the final suspending fluid. Blood was used fresh from single donors who had not ingested aspirin during the week before bleeding. Platelet counts were performed by the method of Brecher and Cronkite (10).

*Preparation of Serum Substrates.*—Normal human donors were bled by clean venipuncture using plastic syringes. The blood was placed into sterile plastic tubes on ice without anticoagulant and immediately centrifuged at 4000 $g$ at 4°C for 20 min to yield native platelet-poor plasma. This was transferred to a sterile glass tube and permitted to clot at room temperature. Platelet counts were performed on the platelet-poor plasma. After sterile removal of the fibrin clot, the residual serum, designated "plasma serum" (11), was used for chemotaxis studies. Whole blood, clotted directly in sterile glass tubes, was used to provide "blood serum."

*Chemotaxis Studies.*—The same donor provided the polymorphonuclear leukocytes (PMN)[1] and serum for any single experiment. Blood for PMN leukocyte preparation was mixed with 10 U of heparin and 0.075 ml of methyl cellulose per ml of whole blood and permitted to settle by gravity in sterile plastic tubes at room temperature. After 1 h the leukocyte-rich supernatant plasma was removed and centrifuged in siliconized tubes at 77 $g$ for 10 min to sediment the PMN leukocytes. Residual red cells were removed by hypotonic lysis. The leukocytes were twice washed with Gey's buffered salt solution containing 0.1% bovine serum albumin, pH 7.0, and resuspended in this medium at a concentration of 2 × 10$^6$ PMN/ml.

*Generation of Chemotactic Activity.*—Chambers modified from the original Boyden type were used, fitted with micropore filters of 3.0 μm pore size. Mixtures of 0.15 ml of serum, 0.1 ml of platelet protein, and 0.05–0.1 ml of buffer were incubated in sterile plastic tubes with agitation at 37°C for a desired period (usually 30 min) and were diluted fivefold with cold buffered Eagle's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, N. Y.). The lower compartment of the chemotaxis chambers was filled with the diluted incubation mixture and the upper compartment with 1 × 10$^6$ PMN. All tests were performed in duplicate. After incubation of the chambers for 2½ h at 37°C in moist air, the filters were removed, fixed in methanol, stained with hematoxylin, dehydrated, and cleared. Cell counts were made of PMN that had migrated entirely through the filter to the lower or far side, and were expressed as the mean of counts of two duplicate filters, with five random high-power fields counted per filter.

*Platelet Aggregation.*—Samples of washed platelets were aggregated with recording by the turbidimetric method of Born, using a Chrono-Log aggregometer (Chrono-Log Corp., Broomall, Pa.) and a Bausch and Lomb VOM-5 recorder (Bausch and Lomb, Inc., Rochester, N. Y.).

*Inhibitors and Special Reagents.*—Collagen, adenosine diphosphate (ADP), soybean trypsin inhibitor, ethylenediaminetetraacetic acid, tosyl arginine methyl ester (TAMe), benzoyl arginine methyl ester (BAMe), glycyl glycine ethyl ester (GGEE), and epsilon aminocaproic acid (EACA) were purchased from Sigma Chemical Corp. (St. Louis, Mo.). Purified alpha-2-macroglobulin from human plasma was the gift of Dr. Peter Harpel, Cornell University Medical College. Purified human C3 and C5 were kindly provided by Dr. Margaret Polley of Cornell University Medical College. Antibodies to C3 and C5 were purchased from Cordis Laboratories Inc. (Miami, Fla.).

## RESULTS

The intrinsic chemotactic activity of serum was evaluated to determine the possible contribution made by platelets during clotting. Serum prepared from

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[1] *Abbreviations used in this paper:* ADP, adenosine diphosphate; MEM, Eagle's minimal essential medium; PMN, polymorphonuclear leukocytes.

clotted whole blood (here termed blood serum) has been observed by other investigators to possess chemotactic activity without the addition of any known chemotactic agent (12). Blood was therefore drawn into chilled plastic tubes and immediately centrifuged to sediment all erythrocytes, leukocytes, and platelets before the supernatant plasma was removed and permitted to clot. Serum prepared in this manner (here termed plasma serum) had little, if any, chemotactic activity (Fig. 1). As the platelet count in the centrifuged plasma was increased by slower centrifugation, the chemotactic activity proportion-
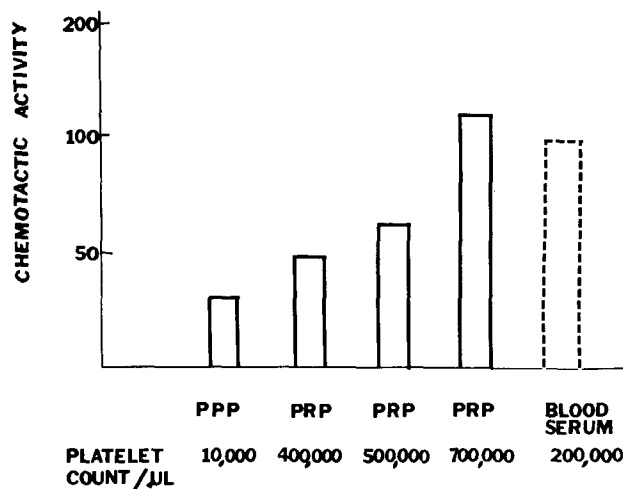


Fig. 1. The chemotactic activity of serum derived from plasmas containing varying platelet concentrations compared with the activity of whole blood serum. Chemotactic activity denotes the total number of polymorphonuclear leukocytes that have migrated completely through a 3 μm pore filter after 2½ h incubation at 37°C, as counted over five high-power microscope fields. Each bar represents mean of two duplicate assays using serum with designated platelet count. This figure depicts results of a single typical experiment using blood from a single normal subject. PPP, platelet-poor plasma; PRP, platelet-rich plasma.

ately increased in the resultant serum. In order to evaluate the contribution of platelet constituents to serum chemotactic activity, the serum used for subsequent experiments was prepared from platelet-poor plasma (plasma serum).

Suspensions of washed platelets and platelet fractions alone possessed no chemotactic activity for polymorphonuclear leukocytes in the absence of serum. However, upon incubation of platelet protein fractions with plasma serum, marked chemotactic activity appeared (Fig. 2). Platelet granule lysates were significantly more active than whole platelet extracts.

Washed platelets, suspended in buffer solution, were aggregated to determine whether the factor that generates chemotactic activity in serum is released from the cells (Table I). The aggregating agents collagen, epinephrine, and ADP alone were inactive. Supernatant fluid from suspensions of platelets ag-

gregated with collagen and epinephrine, after sedimentation of the platelet aggregates, generated chemotactic activity in serum. The supernatant fluid from nonaggregated platelets or from platelets reversibly aggregated with ADP did not generate chemotactic activity.
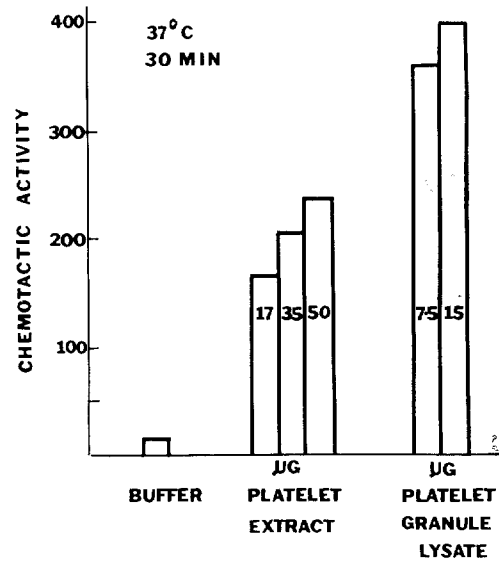


FIG. 2. Chemotactic activity generated in plasma serum by platelet protein fractions. Each bar denotes the chemotactic activity produced by the designated amount of platelet protein incubated with 0.15 ml of fresh plasma serum for 30 min at 37°C and diluted five-fold with buffered MEM for chemotactic assay. The bar marked buffer indicates activity of serum incubated only with phosphate-buffered saline.

TABLE I

*Release of Chemotaxis-Generating Factor from Washed Platelets by Aggregation*

| Supernatant from platelets* treated with | Aggregation | Chemotactic activity‡ |
| --- | --- | --- |
| Buffer | None | 15 |
| ADP, collagen, or epinephrine without platelets | None | 30 |
| ADP 1 $\mu$M | Reversible | 35 |
| Collagen 10 $\mu$l (4 mg/ml) | Yes | 278 |
| Epinephrine 10 $\mu$M | Yes | 220 |

* Platelets washed in Ardlie buffer system, suspended at 200,000/$\mu$l.

‡ Generated by incubation of 0.1 ml of platelet supernatant or aggregating agent, 0.15 ml of serum, and 0.05 ml of buffer for 30 min at 37°C. Activity represents mean number of PMN per five high-power fields that have migrated through a 3 $\mu$m pore filter. Mean value of duplicate filter counts.

*Factors Influencing the Generation of Chemotactic Activity by Platelet Protein.—*

*Temperature:* The generation of chemotactic activity in fresh serum by platelet protein was dependent on the temperature of incubation (Fig. 3). Very little activity appeared after incubation at 0°C, while activity increased with increased temperatures up to 37°C.

*Duration of incubation:* During incubation at 37°C, the chemotactic activity generated increased with incubation time to a maximum at 30 min (Fig. 4). Longer incubation periods resulted in a diminution of activity.

*pH of incubation:* The generation of chemotactic activity in serum by platelet protein also was dependent upon pH, with maximal activity generated in the neutral range, pH 7.2–7.4 (Fig. 5). No activity was generated at pH 3.8–4.0, the optimum range for many lysosomal cathepsins (13).

*Stability of platelet protein:* The chemotaxis-generating capacity of human platelet acid extract was stable for weeks when stored at acid pH and 4°C or at neutral pH at $-20$°C, but was lost at neutral pH upon storage at room temperature. The activity was stable to heating to 100°C for 10 min.

*Role of inhibitors:* Exposure of the platelet extract to the enzyme inhibitors alpha-2-macroglobulin, soybean trypsin inhibitor, and epsilon aminocaproic acid (EACA) rapidly abolished the capacity to generate chemotactic activity in serum (Table II). Once activity was generated in serum the addition of these substances had little or no effect. Treatment of the platelet extract with tosyl argininine methyl ester (TAMe) or benzoyl arginine methyl ester (BAMe) partially inhibited its subsequent chemotaxis-generating activity, whereas glycyl glycine ethyl ester (GGEE) was without significant effect (Table II).
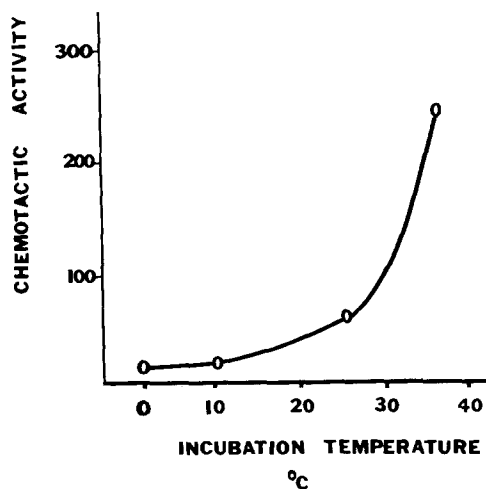


Fig. 3. Effect of incubation temperature on generation of platelet-dependent chemotactic activity in serum. Each point represents chemotactic activity produced after 30 min incubation of 40 μg of human platelet acid extract and 0.15 ml of plasma serum at pH 7.2.

*Optimum proportions of platelet protein to serum substrate:* Maximal chemo-tactic activity was dependent upon the ratio of platelet factor to serum (Table III). For a constant amount of serum, increasing chemotactic activity was generated by incubation with increasing amounts of platelet protein up to 40–50 μg
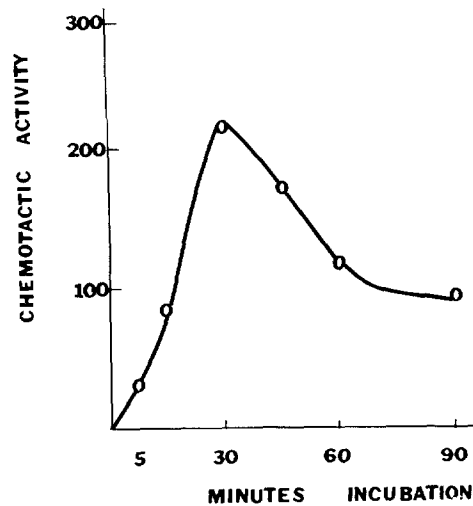


FIG. 4. Effect of duration of incubation on generation of chemotactic activity. Each point represents mean chemotactic activity generated during incubation of 40 μg of human platelet acid extract and 0.15 ml of plasma serum at pH 7.2. Incubations were terminated by fivefold dilution with MEM and chilling in ice until chemotactic assay.
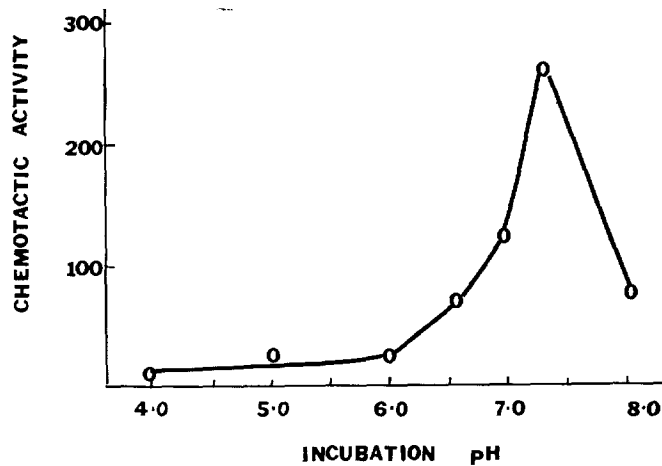


FIG. 5. Effect on incubation pH on generation of chemotactic activity. Each point represents the mean chemotactic activity generated during incubation of 40 μg of human platelet acid extract and 0.15 ml of plasma serum at indicated pH for 30 min at 37°C. Incubation was terminated by fivefold dilution with MEM and adjustment of pH to 7.2 for chemotactic assay.

TABLE II

*Effect of Inhibitors on Generation of Chemotactic Activity by Platelet Protein*

| Test substances* | Chemotactic activity generated‡ | Inhibition |
|---|---|---|
| | | % |
| Buffer (no inhibitor) | 363 | — |
| Soybean trypsin inhibitor (0.15 mg/ml) | 71 | 81 |
| Alpha-2-macroglobulin (0.12 mg/ml) | 24 | 93 |
| Epsilon amino caproic acid (0.03 M) | 104 | 71 |
| Tosyl arginine methyl ester (0.03 M) | 43 | 89 |
| Benzoyl arginine methyl ester (0.03 M) | 302 | 16 |
| Glycyl glycine ethyl ester (0.03 M) | 330 | 9 |

* Final concentration in incubation mixture.

‡ 0.15 ml of plasma serum incubated with mixture of test substances and 35 μg of platelet acid extract for 30 min at 37°C.

TABLE III

*Protein Content of Platelet Extract in Relation to Generation of Chemotactic Activity*

| Platelet protein* | Relative chemotactic activity‡ |
|---|---|
| μg/assay | % |
| 10 | 70 |
| 20 | 84 |
| 30 | 87 |
| 40 | 98 |
| 50 | 93 |
| 70 | 83 |
| 100 | 57 |

* Micrograms of platelet acid extract incubated with 0.15 ml of fresh plasma serum for 30 min at 37°C and diluted fivefold for chemotactic assay.

‡ Chemotactic activity at each level tested in a single experiment was expressed as a percent of peak value. The results given above are the average of normalized values from 10 experiments with eight different preparations of platelet protein.

of whole platelet protein per 0.15 ml of serum. Larger amounts of platelet protein tended to yield decreased activity.

*Serum requirements:* Fresh serum supported generation of chemotactic activity by platelet protein extracts. Plasma anticoagulated with heparin or with sodium citrate did not generate activity. If serum was heated to 56°C for 30 min before incubation with platelet extract, no chemotactic activity was produced (Table IV). Treatment of serum with 0.001 M hydrazine, with heparin, or with 1 M KSCN all prevented the generation of chemotactic activity by platelet protein. Aged serum, kept at room temperature overnight or at 10°C for several days, was also inactive. The addition of 0.001–0.01 M disodium EDTA to fresh plasma serum, however, did not interfere with subsequent generation of chemotactic activity but rather enhanced its production.

From the above studies it appeared likely that serum complement activity was related to the platelet-dependent generation of chemotactic activity in the serum. The following studies were carried out to determine the contribution of specific complement components.

Preincubation of serum substrate with specific antibody to C3 and C5 did not generate chemotactic activity (Fig. 6). When antibody-treated serum was incubated with platelet protein, chemotactic activity was readily generated in serum exposed to anti-C3 antibody, but activity was markedly inhibited in serum exposed to anti-C5 antibody. The addition of either anti-C3 or anti-C5 antibody to the serum-platelet protein mixture at the end of the incubation period did not inhibit the newly generated chemotactic property.

To test the requirement for complement directly, purified human C3 and C5 were substituted as the substrate for the platelet protein (Fig. 7) and serum was

TABLE IV

*Effect of Serum Treatment of Chemotactic Activity Generated by Platelet Protein*

| Substrate* | Chemotactic activity‡ | Inhibition |
|---|---|---|
| | | % |
| Fresh plasma serum | 363 | 0 |
| Heated serum (30 min at 56°C) | 7 | 99 |
| Hydrazine-treated serum (0.015 M) | 8 | 98 |
| Serum + EDTA 0.01 M | 403 | Enhanced |

\* 0.15 ml of substrate incubated with 35 μg of platelet extract for 30 min at 37°C, pH 7.2.
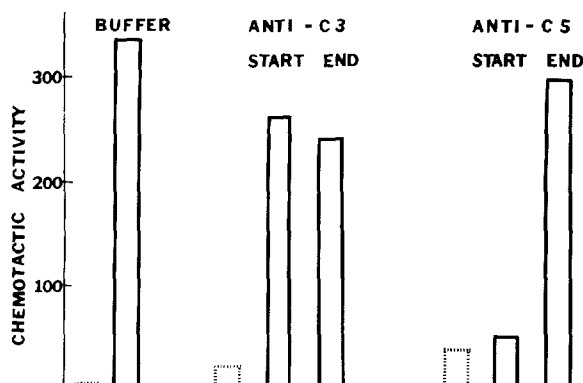‡ Mean cell count per five high-power fields in duplicate filters.



FIG. 6. Effect of anticomplement antisera on generation of platelet-dependent chemotactic factor in plasma serum. Stippled bars represent chemotactic activity generated by treatment of plasma serum with buffer, anti-C3, or anti-C5 in the absence of platelet protein. Solid bars represent chemotactic activity generated in plasma serum by platelet protein after pre-incubation of serum with buffer, anti-C3, or anti-C5 (bars marked "start"). Anti-C3, and anti-C5 were also added to incubation mixture of plasma serum and platelet protein at end of the 30 min incubation at 37°C (bars marked "end").

omitted. Incubation with the individual complement components resulted in the generation of chemotactic activity only from C5.

## DISCUSSION

The mechanisms governing leukocyte chemotaxis, an important early step in the development of inflammatory reactions, have been extensively studied in the past decade. The central role of complement-derived chemotactic factors in inflammation has been clearly defined (14, 15), and the reinforcement of chemotaxis by the inflammatory exudate cells themselves interacting with serum complement has been shown to be a means for amplifying or propagating the inflammatory response. Ward and Hill (16) have demonstrated that an enzyme in the lysosomal granules of PMN leukocytes from rabbits produces a C5-derived chemotactic peptide, while Snyderman, Shin, and Dannenberg (17) have shown that monocytes can generate a similar, perhaps not identical, chemotactic factor from C5. A variety of injured or infected tissues has been shown to liberate a chemotactic fragment from C3 or C5 (18–20).

In previous studies on inflammatory properties of platelets (3, 21, 22) we showed that an acid extract of human platelet granules both induced an increase in vascular permeability in rabbit skin and produced an intense exudation of PMN leukocytes at the injection site. The first, but not the second, phenomenon was related to histamine release. The present investigation was aimed at defining the mechanism of this leukocyte response.

The data presented here indicate that a protein fraction present in human platelets, itself lacking any chemotactic property, can generate chemotactic activity in fresh serum. Much of the "background" or intrinsic chemotactic
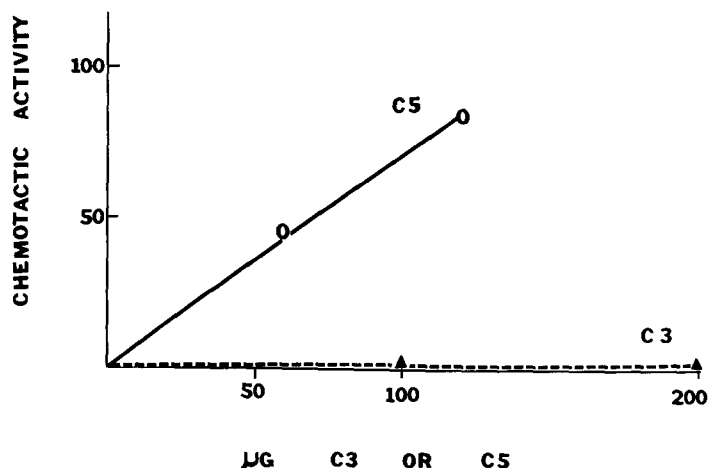


FIG. 7. Generation of chemotactic activity from purified complement components by human platelet acid extract. Circles (○) indicate chemotactic activity generated from purified human C5 by incubation with 50 μg of human platelet acid extract for 30 min at 37°C at pH 7.2. Triangles (▲) indicate results when human C3 was used as substrate.

activity of serum detected by other investigators appears to be related to the presence of platelets during clotting. If cell-free plasma is clotted the chemotactic property of the serum virtually disappears, whereas platelet-rich plasma when clotted yields serum of high chemotactic activity. The factor responsible for production of chemotactic activity in plasma serum is released from washed platelets during aggregation by agents such as collagen and epinephrine, suggesting that it is a normal platelet constituent released under physiologic conditions as part of the platelet release reaction.

The platelet factor that generates chemotactic activity in serum resides in the same acid-extractable protein fraction as the vascular permeability factor previously described. The activity can, similarly, be further localized to the lysosomal granules of platelets. This platelet protein fraction acts upon the fifth component of complement to liberate chemotactic activity for PMN leukocytes in a manner very similar to the C5-cleaving factor from rabbit PMN leukocyte granules described by Ward and Hill (16). Both the platelet factor and the leukocyte C5-cleaving factor have maximum activity at neutral pH and at body temperature and demonstrate similar optima for incubation time and substrate:factor ratios. Inhibitor patterns are also generally parallel.

Studies employing inhibitors of complement, specific anticomplement antisera, and purified complement components indicate that the platelet factor acts directly upon C5 to liberate a chemotactic principle. The generation of chemotactic activity in plasma serum is inhibited by pretreatment with anti-C5 antiserum but not by antibody against C3. EDTA, which inhibits the classical pathway of complement activation (23) and in higher concentration (0.01 M) the alternate mechanism as well (24), does not inhibit the generation of chemotactic activity in serum by the platelet factor. That EDTA actually enhances the expression of chemotactic activity by the platelet-dependent pathway (Table IV) may reflect an EDTA-mediated protection of the chemotactic fragment from complement-inactivating substances present in serum. This enhancing effect of EDTA upon platelet-dependent chemotactic activity contrasts with the inhibition by EDTA of PMN leukocyte granule-dependent generation of chemotactic activity from C5 (16).

The demonstration that human platelets contain and during aggregation release an activity that generates chemotactic fragments from C5 provides another clear parallel between the inflammatory activities of platelets and of PMN leukocytes. This physiologic resemblance reflects the evolutionary heritage of the hemocyte; moreover, it shows that the specialized hemostatic cell and the specialized inflammatory cell share common functional capabilities. Since platelets remain essentially intravascular, their possession of a chemotaxis-generating system offers a platelet-dependent mechanism for the initiation of PMN leukocyte responses to vascular injury. It also provides for generation of chemotactic activity during the process of blood coagulation. This may promote the invasion of clots by leukocytes for clot resolution and lysis.

The existence of this platelet-dependent chemotactic pathway stresses the close relationship between the processes of hemostasis and of inflammation, in both of which complement activation plays a pivotal role. Several recent studies have indicated that complement activation mediates platelet damage and hypercoagulability. Normal blood clotting in the rabbit depends on the presence of the sixth component of complement (25), and the platelet release reaction in response to endotoxin or antigen-antibody complexes is defective in C6-deficient animals (26). In guinea pigs, endotoxin produces platelet damage, thrombocytopenia, and a shortened clotting time if the complement sequence is intact (27). In C4-deficient animals thrombocytopenia or shortened clotting time do not follow after endotoxin injection. Thus, in these two species complement activation is important in the development of inflammatory responses to intravascular stimuli mediated by platelets. The present study suggests that the relationship between platelets and complement in inflammation also proceeds in the opposite direction, so that platelet factors may directly activate complement components.

## SUMMARY

A protein fraction extracted from the lysosomal granules of human platelets generated chemotactic activity for polymorphonuclear leukocytes when incubated with fresh serum. The platelet factor was also released during platelet aggregation with collagen or epinephrine and appeared to be released during blood clotting. Heated serum did not support the platelet-dependent generation of chemotactic activity. Treatment of fresh serum with antibody to the fifth component of complement also prevented development of activity. Purified human C5 but not C3 yielded chemotactic activity upon incubation with the platelet factor. Thus, human platelets are capable of stimulating chemotaxis via complement activation in a manner similar to leukocytes, and may therefore participate in the early stages of inflammation.

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