

RABBIT PLATELET BACTERICIDAL PROTEIN*†

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The bactericidal capacity of normal serum has been of interest since the early studies of Metchnikoff on the inflammatory response. A heat-stable bactericidal system in animal serum, active against Gram-positive organisms, was first described in 1887 by Fodor (1). This activity was named beta lysin, to distinguish it from the alpha lysin or heat-labile antibacterial system involving complement. Gengou, in 1901, demonstrated that the heat-stable bactericidal activity was cell derived (2). When rabbit blood was drawn into chilled, paraffin-lined tubes to delay clotting and all cellular elements were removed by centrifugation before the cell-free plasma was allowed to clot, the serum so produced did not kill bacteria. Serum from clotted whole blood, on the other hand, was strongly bactericidal. The interpretation of this phenomenon was that cells, probably leukocytes, were responsible for the generation of bactericidal activity.

Several lines of evidence have since implicated platelets rather than leukocytes or erythrocytes as the cellular source of the beta lysin bactericidal activity. In 1960 Hirsch extended Gengou's experiments by reporting that the development of the heat-stable bactericidin in rabbit serum required the presence only of blood platelets during clotting of the cell-free plasma; addition of other types of blood cells did not contribute to the bactericidal effect (3). Saline extracts of platelets, however, did not have any antibacterial activity. Hunder and Jacox demonstrated that serum from rabbits made thrombocytopenic by X-irradiation or by administration of antiplatelet serum had a markedly decreased bactericidal capacity (4). Jago and Jacox found that two components were necessary for serum bactericidal activity (5). One was released from platelets by homogenization in saline, but the second was released only after sonication. They noted that these components were relatively heat stable. Myrvik and Leake described extraction of two serum components from rabbit serum by precipitation with low concentrations of ethanol, a procedure which precipitates basic proteins (6). The two components together, but not separately, were bactericidal.

Basic proteins present in lysosomal granules of rabbit polymorphonuclear leukocytes have been shown to participate in inflammatory responses as bactericidal and as

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permeability-enhancing factors (7, 8). Normal human platelets contain similar basic proteins which are not bactericidal but are associated with enhancement of vascular permeability (9). Rabbit platelets have not been studied previously for their content of basic proteins.

The studies reported here were designed to determine whether the heat-stable bactericidal activity of rabbit serum is related to platelet cationic proteins. Our observations indicate that cationic proteins which are present in platelet lysosomal granules have bactericidal activity. These proteins are released from the platelets during the process of aggregation and represent the source of the rabbit serum bactericidin.

Materials and Methods

Preparation of Rabbit Platelet Acid Extract.—Healthy, outbred, adult New Zealand White rabbits were used for all studies. Rabbits were bled from the ear artery by plastic cannula (Medicut, A. S. Aloe Co., St. Louis, Mo.) into plastic tubes containing 0.1 vol of 3.8% trisodium citrate. Platelet-rich plasma (PRP)¹ was separated by centrifugation at 250 g for 30 min at room temperature in an International model UV centrifuge (International Equipment Company, Needham Heights, Mass.), and the platelets were sedimented from the PRP in siliconized oil bottles by centrifugation at 1000 g for 30 min at room temperature. Contamination of platelets by leukocytes was less than 1 cell/10⁴ platelets. The platelet buttons were pooled and washed six times in Alsever's solution and four times in Gaintner buffer. Washed platelets were resuspended in 4 vol of 0.2 N H₂SO₄ and stirred overnight at 4°C, or were sonicated for 30 sec in normal saline using a Branson sonifier (Branson Instruments, Inc., Stamford, Conn.) (4 amp, setting 4) before acid extraction. The rabbit platelet acid extract (RPAE) was dialyzed against 0.01 M phosphate-buffered normal saline at pH 5.6 before use in bacteriologic assays. For preparation of subcellular fractions, washed platelets were suspended in 0.44 M sucrose containing 0.001 M ethylenediaminetetraacetate (EDTA), homogenized in the cold, and subjected to sucrose density gradient ultracentrifugation by the method of Marcus et al. (10). All preparations were sterilized by filtration through a 0.45 μ pore membrane filter (Millipore Corp., Bedford, Mass.) before use.

Bactericidal Assay.—*Bacillus subtilis* was grown for 18 hr at 37°C with shaking in Trypticase soy broth (Baltimore Biological Laboratories, Baltimore, Md.) containing 1% added dextrose. The bacteria were collected by centrifugation and resuspended in 0.15 M saline containing 0.1% bovine serum albumin (Pentex Biochemical, Kankakee, Ill.). A stock suspension of bacteria was prepared by adjustment to an optical density at 650 nm of 0.270, and a 1:1000 dilution in saline-albumin solution was used to inoculate test samples. Platelet acid extract dialyzed against buffered saline was serially diluted in saline-albumin solution in sterile plastic test tubes. After inoculation with *B. subtilis* the tubes were incubated at 37°C with mixing on a Lab Tek aliquot mixer (Ames Co., Inc., Elkhart, Ind.) for 1 hr. Aliquots were then pipetted into Petri dishes and pour plates were made with melted Trypticase soy agar. Surviving colonies were counted after overnight incubation. Duplicate samples agreed within 10%. A 50% reduction in bacterial colony count was considered significant killing.

Platelet Aggregation.—Aggregation was tested in two systems: in platelet-rich plasma and in suspensions of washed platelets. Fresh, citrated, rabbit platelet-rich plasma was adjusted

¹ Abbreviations used in this paper: PF 4, platelet factor 4; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RPAE, rabbit platelet acid extract.

to a platelet count of 500,000/mm³ with platelet-poor plasma (PPP). Rabbit platelets were washed three times in Alsever's solution at room temperature and resuspended in Gey's buffered salt solution, pH 7.0, containing 0.35% bovine albumin and 1% dextrose. Soluble collagen² (11) (4 mg/ml in 0.5 M CaCl₂), bovine thrombin (Parke, Davis & Company, Detroit, Mich.), and adenosine diphosphate (ADP) (Pabst Research Laboratories, Milwaukee, Wis.) (2×10^{-6} M) were added to 1.2-ml samples of the platelet preparations and aggregation was observed and recorded on an Aggregometer (Chrono-log Corp., Broomall, Pa.). Immediately after aggregation of the platelet preparation, the supernatant was separated by centrifugation at 1000 g and either assayed for bactericidal activity directly or extracted with 0.2 N H₂SO₄ and then assayed.

Platelet Factor 4 Assay.—Antiheparin activity (platelet factor 4) of the platelet extract was measured using the thrombin clotting time method of Poplawski and Niewiarowski (12). The test system comprised 0.3 ml fresh citrated rabbit PPP, 0.1 ml saline or platelet extract, 0.1 ml heparin (0.2 units/ml), 0.1 ml of bovine thrombin (10 units/ml) was added and clotting time was recorded during mixing at 37°C. Heparin concentration was selected to yield a clotting time at least three times that of a saline-PPP control. Partially purified platelet factor 4 was prepared by the zinc acetate precipitation method of Niewiarowski et al. (13).

Characterization and Purification of Rabbit Platelet Acid Extract.—Rabbit platelet acid extract dialyzed against 0.02 M sodium acetate buffer, pH 6, was applied to diethylaminoethyl (DEAE) cellulose columns equilibrated with the same buffer. In some studies the buffer system included 0.05 M NaCl. 2-ml fractions were collected with a Buchler fraction collector (Buchler Instruments, Inc., Fort Lee, N. J.) equipped with an LKB Uvicord recorder (LKB Instruments, Inc., Rockville, Md.). For elution, a gradient of 0–0.5 M NaCl in 0.02 M sodium acetate buffer, pH 6, was applied. Samples of representative fractions were adjusted to pH 5.6 with 0.01 M NaOH, sterilized by filtration, and assayed for bactericidal activity and for platelet factor 4 activity. The active material from the DEAE columns was concentrated by ultrafiltration and subjected to gel filtration on G-75 Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden). The molecular weight of effluent peaks was estimated by protein markers of known molecular weight. Active fractions were concentrated and analyzed by acrylamide gel electrophoresis using the Reisfeld buffer system (14).

Assay for lysozyme was performed by radial diffusion of RPAE in agar containing non-viable *Micrococcus lysodeikticus*, using normal serum containing known concentrations of lysozyme as control. Pepsin digestion of the RPAE was performed according to the method of Bailey et al. (15).

RESULTS

The protein fraction extracted from washed rabbit platelets by dilute H₂SO₄ killed *Bacillus subtilis* suspended in saline-albumin solution. The bactericidal activity was directly related to the protein concentration of the RPAE (Table I). Concentrations of 0.5 μg protein/ml, or greater, killed more than 50% of the bacterial inoculum (1×10^3 – 5×10^5 organisms/ml). Although different preparations of rabbit platelet extracts varied in bactericidal activity, all demonstrated a similar range with greater than 90% killing at concentrations of 5 μg/ml.

Species sensitive to the bactericidal effect of RPAE included several strains of *Staphylococcus albus* and one strain of *S. aureus* (giorgio). Gram-negative

² Kindly supplied by Dr. Albert Rubin, Cornell University Medical College.

organisms tested (*Escherichia coli* and *Salmonella newport*), as well as most strains of *S. aureus*, were not susceptible to killing by RPAE. The bactericidal spectrum of RPAE is essentially the same as that reported for rabbit serum by Hirsch (3).

The bactericidal effect of the RPAE was rapid (Fig. 1). Significant killing of stationary-phase cultures (18 hr growth) occurred within 30 min of incubation of bacteria with RPAE and was complete after 90 min. The rates of killing were similar at concentrations of 10^2 – 10^5 bacteria/ μ g of platelet protein. The fraction of bacteria surviving was slightly greater with larger inocula. Bacteria in the logarithmic phase of growth (4 hr) were killed more completely and rapidly than bacteria from stationary-phase cultures, with greater than 90% killing achieved by 10 min exposure. The bactericidal effect of the RPAE

TABLE I
*Bactericidal Activity of Rabbit Platelet Acid
Extract Related to Protein
Concentration*

μ g protein/ml	% colonies surviving*
10	4
5	10
2.5	13
1.25	23
0.6	48
0.3	69
0.15	95

* 1 hr incubation at 37°C.

demonstrated temperature dependence (Table II). The maximal killing occurred when incubation with RPAE was carried out at 37°C. Cold was inhibitory, with more than 16 times as much RPAE necessary to produce significant killing at 4°C. The bactericidal activity was adsorbed from the platelet extract by exposure to high concentrations of heat-killed bacteria (Table III). The bacteria were not agglutinated by the RPAE.

Platelet-free rabbit plasma, clotted to form serum, had no significant bactericidal activity when diluted more than 1:5 (Fig. 2). Normal rabbit serum prepared from whole blood was bactericidal at dilutions of 1:40–1:80. RPAE contained from 5 to 10 μ g of protein/ml extracted from rabbit serum. The addition of 1–20 μ g RPAE protein/ml to diluted serum prepared from platelet-free plasma restored the bactericidal activity found in normal rabbit serum. The bactericidal capacity of the RPAE was neither enhanced nor inhibited in the presence of serum.

Properties of the Rabbit Platelet Bactericidal Extract.—The bactericidal activ-

ity of the RPAE was nondialyzable (Table III). The activity was optimal over the pH range of 5.6–7.2. Bacterial growth was itself inhibited by incubation at more acid or alkaline pH conditions. The biologic activity of the RPAE was stable to heating to 56°C for 30 min or to 80°C for 15 min but activity was lost after prolonged boiling. RPAE retained full activity for prolonged periods

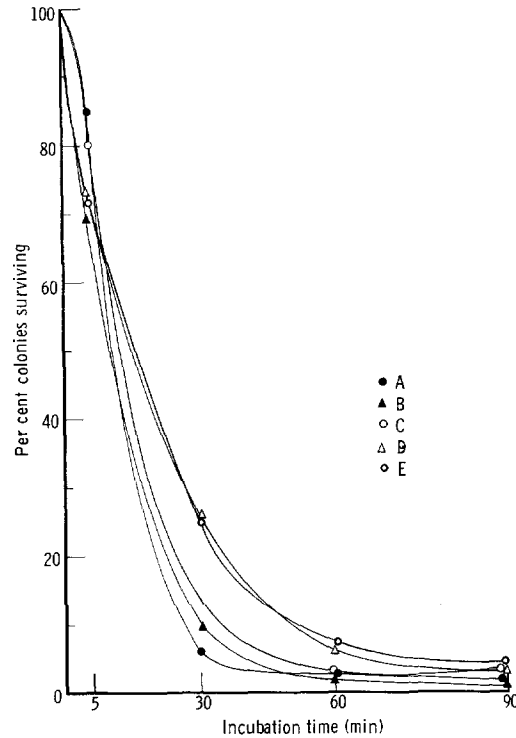


FIG. 1. Bactericidal effect of rabbit platelet acid extract as a function of incubation period. Colonies of *B. subtilis* surviving 5, 30, 60, and 90 min incubation with RPAE at 37°C at different bacterial concentrations. A, 3×10^8 bacteria/ml; B, 6×10^8 bacteria/ml; C, 1×10^4 bacteria/ml; D, 1.5×10^4 bacteria/ml; E, 6×10^4 bacteria/ml. RPAE concentration, 5 μ g/ml.

when kept at low pH (in dilute H_2SO_4) but activity was lost rapidly upon storage at alkaline pH and at low ionic strength, e.g. when dialyzed against 0.01 M sodium phosphate buffer without saline, or when dialyzed against distilled water. In the presence of 0.15 M NaCl activity was retained during storage at $-20^\circ C$ for several weeks. Pepsin digestion resulted in complete loss of bactericidal activity. No lysozyme could be demonstrated in the RPAE.

Bactericidal activity was precipitated from the RPAE by addition of ethanol to a final v/v concentration of 20%. This precipitate, redissolved in saline,

demonstrated bactericidal activity against *B. subtilis* at a concentration of 0.3 μg protein/ml.

Heparin inhibited the bactericidal effect of RPAE at concentrations as low as 0.1 unit/ μg of platelet protein (Table IV). The concentration of RPAE used in the heparin inhibition experiments was 17 times that required to produce 90% bacterial killing. RPAE excess was utilized in these studies in order

TABLE II
*Effect of Incubation Temperature on
Bactericidal Activity of Rabbit
Platelet Acid Extract*

Incubation temperature	% activity
37°C	100
25°C	25
4°C	6

TABLE III
Properties of Platelet Bactericidal Protein

Treatment of RPAE	Bactericidal activity
	% control
Dialysis vs. 0.01 M phosphate-0.15 M NaCl, pH 5.6 (control)	100
Dialysis vs. 0.01 M phosphate-0.15 M NaCl, pH 7.2	100
Dialysis vs. 0.01 M phosphate, no saline, pH 7.2	6
Dialysis vs. H ₂ O	10
Dialysate, concentrated	0
Heating to 80°C, 15 min	100
Heating to 100°C, 30 min	0
Pepsin (0.2%) digestion	0
Exposure to 10 ⁸ killed <i>B. subtilis</i> /ml	0

to emphasize the marked inhibition of bactericidal activity by polyanionic heparin.

Subcellular Localization of Bactericidal Material.—Platelet granules were separated from other components of homogenized platelets by sucrose density gradient ultracentrifugation. Acid extracts of the granule fraction demonstrated strong bactericidal activity (Table V). The membrane fraction was inactive even at high protein concentration.

Release of Bactericidal Activity During Platelet Aggregation.—Bactericidal activity was released when rabbit platelets were aggregated in citrated platelet-rich plasma. Washed platelets suspended in buffered saline also released bactericidal activity after aggregation by collagen (Fig. 3). The bactericidal

material appeared in the supernatant plasma or saline after sedimentation of the aggregated platelets. Some bactericidal activity was present in low dilutions of platelet-poor plasma after centrifugation, probably reflecting mechanical damage to and release from platelets during the centrifugation procedure.

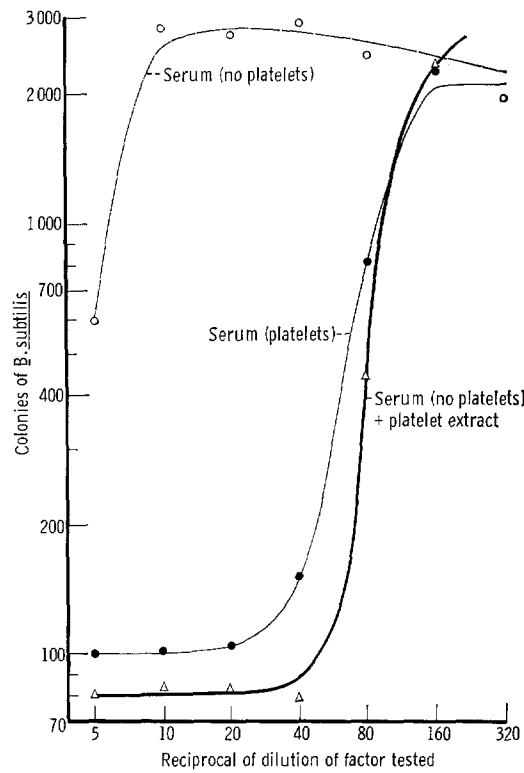


FIG. 2. Bactericidal activity of normal rabbit serum prepared from platelet-free plasma, and rabbit platelet acid extract. Bacteria surviving incubation with: ●—●, dilutions of normal rabbit serum; ○—○, dilutions of serum prepared from platelet-free plasma; △—△, dilutions of RPAE, 120 $\mu\text{g}/\text{ml}$, in the presence of platelet-free serum.

TABLE IV
Heparin Inhibition of Bactericidal Activity of Rabbit Platelet Acid Extract

Heparin <i>units/ml</i>	% colonies surviving*
0	4
0.1	11
1.0	40
10.0	100

* Incubation 1 hr at 37°C using RPAE, 8 $\mu\text{g}/\text{ml}$.

TABLE V
Subcellular Localization of Bactericidal Activity within Platelets

Platelet fraction	Protein concentration $\mu\text{g/ml}$	Surviving colonies of <i>B. subtilis</i>		
		No. of colonies	Control	% survival
Granule	10	2	701	0.3
Membrane	90	632	701	90.1

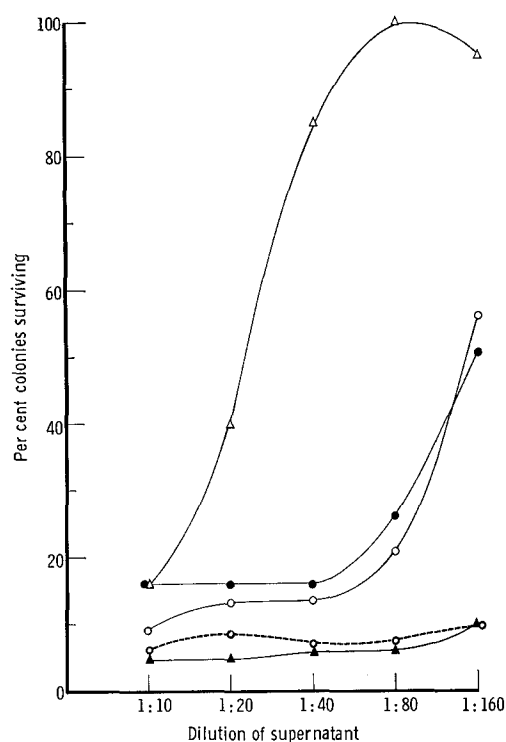


FIG. 3. Release of bactericidal activity from platelets during aggregation, survival of bacteria after incubation with dilutions of supernatant plasma or saline after platelet aggregation by collagen, and sedimentation of platelets at 1000 *g*. Δ — Δ , platelet-poor plasma; \blacktriangle — \blacktriangle , platelet-rich plasma (500,000 platelets/ mm^3) plus 0.4 mg collagen; \circ — \circ , platelet-rich plasma and aspirin (1 mg/ml) plus 0.4 mg collagen; \bullet — \bullet , platelet-rich plasma and imipramine (1.25 mg/ml) plus 0.4 mg collagen; \circ — \circ , washed platelets in Gey's solution plus collagen, 0.4 mg.

Aggregation inhibitors such as imipramine and aspirin significantly decreased the release of bactericidal activity from platelets exposed to aggregating doses of collagen (Fig. 3). Acid extracts of the supernatant fluid remaining after sedimentation of aggregated platelets retained the bactericidal activity.

Collagen-induced platelet aggregation released bactericidal activity from platelets in proportion to the concentration of collagen used (Fig. 4). The upper panel of Fig. 4 depicts the aggregation curves recorded by the platelet Aggre-

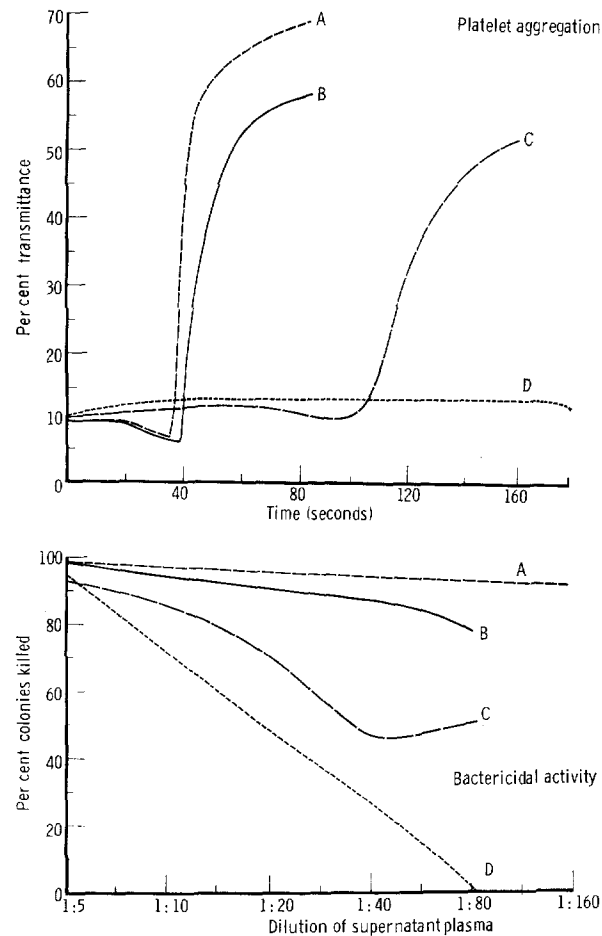


FIG. 4. Release of bactericidal activity from platelets as a function of degree of platelet aggregation by collagen. Upper panel, aggregometer tracing after addition of: *A*, collagen, 0.4 mg; *B*, collagen, 0.1 mg; *C*, collagen, 0.01 mg; and *D*, collagen, 0.1 mg after imipramine (1.25 mg/ml). Lower panel, bacterial killing by supernatant plasma after aggregation and sedimentation of platelets.

gometer for three concentrations of collagen and for collagen in the presence of the inhibitor, imipramine. The greater the protein concentration of collagen used, the greater was the aggregation reaction monitored. The lower panel of

Fig. 4 shows the bactericidal activity released into supernatant plasma at the end of the aggregation reaction. The degree of bactericidal activity similarly corresponded to the collagen concentration and to the degree of platelet aggregation. When collagen-induced platelet aggregation was inhibited by imipramine, the release of bactericidal activity was no greater than the activity in

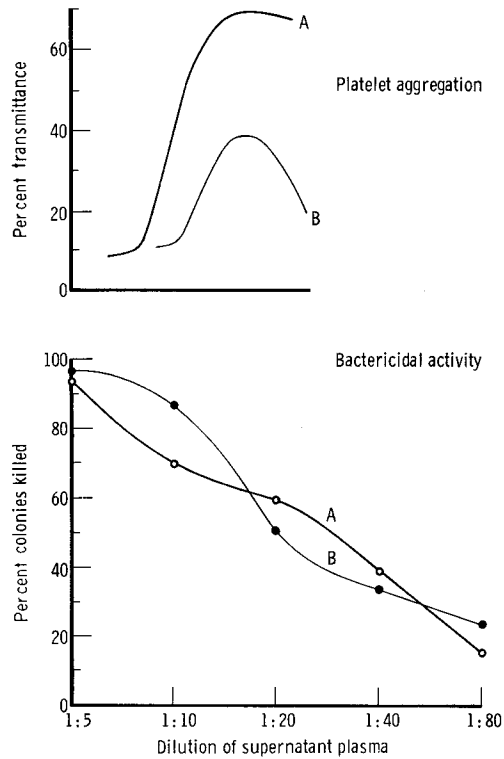


FIG. 5. Effect of adenosine diphosphate-induced platelet aggregation on release of bactericidal activity. Upper panel, platelet aggregation by: *A*, 2×10^{-6} M ADP, and *B*, 5×10^{-7} M ADP (reversible aggregation). Lower panel, bactericidal activity of supernatant plasmas after aggregation and sedimentation of platelets.

platelet-poor plasma alone. Release of bactericidal activity, therefore, closely paralleled platelet aggregation by collagen. Adenosine diphosphate-induced platelet aggregation was associated with only minimal release of the bactericidal activity into the supernatant (Fig. 5). At a low concentration, ADP produced reversible platelet aggregation which was not associated with significant release of bactericidal activity. Thrombin-induced aggregation of washed platelets released bactericidal activity similar to that released by collagen.

Purification of Bactericidal Material.—The rabbit platelet acid extract was partially purified by chromatography on DEAE cellulose (Fig. 6). Bactericidal activity was confined to a small protein peak which was not retarded. The re-

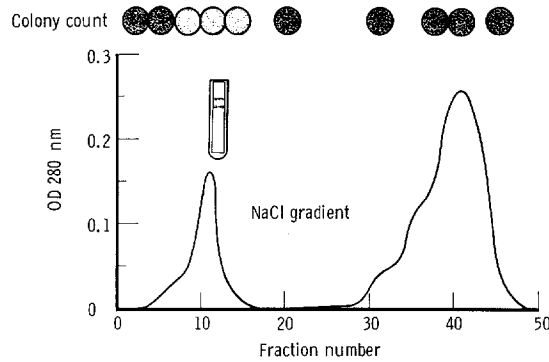


FIG. 6. Partial purification of rabbit platelet acid extract by chromatography on DEAE cellulose. Bactericidal activity depicted by Petri dishes confined to early protein peak. Acrylamide gel electrophoresis reveals two protein bands.

TABLE VI
*Antiheparin Activity of Different Platelet Protein Fractions**

Platelet fraction added	Thrombin clotting time
	<i>sec</i>
None (saline)	15 (no heparin)
None (saline)	49 (0.2 units heparin/ml)
Rabbit platelet acid extract, 15 μ g	22
Bactericidal fraction from DEAE column, 10 μ g	18
20% ethanol precipitate of RPAE, 2 μ g	21
RPAE after removal of 20% ethanol precipitate, 15 μ g	52
Platelet factor 4, 12 μ g	15
PF 4-depleted RPAE	49

* Antiheparin activity was tested by adding fractions to a thrombin clotting time system in which clotting time in seconds was prolonged by addition of heparin sufficient to yield three times baseline clotting time (seconds). The test system consisted of 0.3 ml fresh rabbit citrated, platelet-poor plasma, 0.1 ml test material, and 0.1 ml of heparin (0.2 units/ml); 0.1 ml thrombin (10 units/ml) was added as the stopwatch was started. All materials were incubated at 37°C and all tests were performed in duplicate.

mainder of the protein, which was eluted with a gradient of NaCl, had no antibacterial activity nor did it alter the activity of the bactericidal peak when recombined with the latter. Cationic acrylamide gel electrophoresis of the bactericidal peak revealed two protein bands.

Gel filtration of the bactericidal peak taken from DEAE cellulose was carried

out using Sephadex G-75. Two peaks were detected, of approximately 40,000 and 10,000 mol wt. Both of these peaks demonstrated bactericidal activity.

Antiheparin Activity and Rabbit Platelet Acid Extract.—The rabbit platelet acid extract demonstrated antiheparin activity similar to that of platelet factor 4 when tested in a thrombin clotting time system (Table VI). That is, RPAE added to a mixture of fresh plasma and heparin counteracted the prolongation of thrombin time produced by heparin. The bactericidal peak from DEAE cellulose chromatography of RPAE also possessed antiheparin activity. Platelet factor 4, prepared directly from washed rabbit platelets, demonstrated two

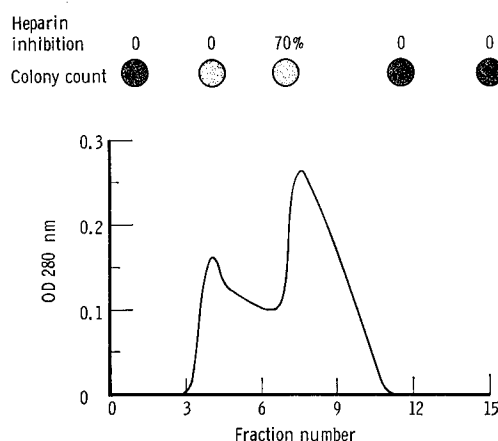


FIG. 7. Sephadex G-75 gel filtration of bactericidal protein peak from DEAE cellulose chromatography. Separation of bactericidal and platelet factor 4 activities.

to three times as much antiheparin activity per microgram of protein as did the RPAE preparations.

Separation of the platelet factor 4 activity from the bactericidal activity of the RPAE was achieved by several methods. Separation of the two activities was first accomplished by ethanol precipitation. The fraction of RPAE precipitated by 20% (v/v) ethanol demonstrated antiheparin activity (Table VI). This fraction was also bactericidal. However, the residual portion of the RPAE not precipitated by 20% ethanol was equally bactericidal but lacked antiheparin activity. Zinc acetate precipitation was used to deplete the RPAE of platelet factor 4 (PF 4). The PF 4-depleted RPAE remained fully bactericidal but lost all antiheparin activity. Finally, separation of platelet factor 4 and bactericidal activities was achieved by Sephadex gel filtration of the DEAE cellulose bactericidal peak (Fig. 7). Fig. 7 demonstrates that two peaks were eluted from the Sephadex gel column after application of the DEAE cellulose bactericidal peak. The fraction of mol wt 10,000 possessed antiheparin activity. The fraction of mol wt 40,000 lacked antiheparin activity.

Platelet factor 4, prepared according to the method of Niewiarowski, was moderately bactericidal to *B. subtilis*, but required a protein concentration of 30 $\mu\text{g}/\text{ml}$ or greater for significant bactericidal effect, whereas an acid extract of the platelet residue after removal of PF 4, entirely lacking in antiheparin activity, was strongly bactericidal.

DISCUSSION

These studies demonstrate that the heat-stable antibacterial activity of rabbit serum not only is platelet dependent, but resides preformed within the platelets as one or more cationic protein constituents of platelet lysosomal granules. The platelet bactericidal proteins bear significant resemblance to the antibacterial proteins found in blood leukocytes by virtue of their cellular localization in granules, heat stability, absence of dependence on serum complement, positive charge, and inactivation by anionic materials. Whereas leukocyte bactericidal proteins are released into intracellular vacuoles during phagocytosis, the platelet bactericidal proteins are released into the bloodstream during platelet aggregation and during blood coagulation.

Characterization of the platelet bactericidal protein fraction by ion exchange chromatography and gel filtration has revealed a low molecular weight protein of strongly positive charge which appears distinct from the antiheparin factor, platelet factor 4. This protein is much less stable than platelet factor 4 under conditions of low ionic strength and alkaline pH. In these characteristics the platelet bactericidal protein also closely resembles the leukocyte bactericidal proteins.

The crucial mechanism in the development of serum antibacterial activity against Gram-positive microorganisms is the platelet aggregation which occurs concomitantly with blood coagulation, rather than coagulation per se. Blood coagulation involves the activation and cascading interaction of a series of protein precursors present in the blood plasma; platelets normally play a catalytic role by providing a specialized surface (platelet factor 3) which accelerates this series of fluid-phase reactions to form a blood clot. Coagulation may, however, occur normally in the presence of severe thrombocytopenia. During injury to a blood vessel, exposure of subendothelial collagen acts as an initiator both to coagulation and to the aggregation of platelets so that the two processes normally occur together (16). Platelet aggregation may take place quite independently of blood clotting. In vitro, evidence that the release of antibacterial activity depends only upon platelet aggregation and not on coagulation is derived from the observations that (a) washed platelets suspended in saline release bactericidal protein upon aggregation in the absence of plasma; (b) the coagulation of platelet-free plasma does not yield bactericidal activity in the serum produced; and (c) platelet aggregation in anticoagulated platelet-rich plasma releases bactericidal activity although clotting does not occur.

The studies reported here suggest that the release of bactericidal activity from platelets requires irreversible platelet aggregation by surface active agents. Thus collagen and thrombin but not small doses of ADP produce release of bactericidal activity. It would appear that both aggregation and the platelet release reaction involving platelet degranulation are prerequisites for liberation of bactericidal proteins. This is supported by the data showing that the amount of bactericidal activity released is directly related to the degree of platelet aggregation produced by collagen, a surface-active aggregating agent (Fig. 4). Inhibitors of platelet aggregation effectively block the release of bactericidal activity into the supernatant (Figs. 3 and 4). The minimal effect of ADP-induced aggregation in releasing bactericidal activity also supports the concept that this granule-bound component only leaves the platelet under conditions of profound platelet disruption, for rabbit platelets exhibit only reversible aggregation on exposure to ADP (17).

The relationship between the platelet bactericidal protein and platelet factor 4 (PF 4) is a close one. PF 4 is a low molecular weight, positively charged protein which possesses marked antiheparin activity. It is released by saline extraction of frozen-thawed platelets and by platelet aggregation. It is stable to heating and to storage and is precipitable by zinc ions. No previous assay of PF 4 for antibacterial activity has been reported. We have found that PF 4 has a modest bactericidal effect on *B. subtilis* at high protein concentrations. The crude rabbit platelet acid extract possessed PF 4 activity. However, after precipitation of PF 4 with zinc ions, the platelet acid extract retained strong bactericidal activity. Conversely the platelet residue after extraction of PF 4 possessed antibacterial activity which was acid extractable. This suggests that crude rabbit platelet acid extract contained a different and more potent bactericidal material as well as PF 4. Both activities appeared together in the cationic bactericidal peak on DEAE cellulose, as might be expected for two basic proteins. The two activities were separable by gel filtration on Sephadex. Many positively charged proteins and peptides have been reported to demonstrate antibacterial activity, and this may account for the bactericidal activity associated with PF 4. However, the data reported here indicate that the lysosomal bactericidal protein of platelets is indeed separable from platelet factor 4.

The bactericidal effect of the rabbit platelet cationic protein suggests that the bacteria need to be metabolically active. This is indicated by the greater killing effect at 37°C than at lower temperatures, and by a more rapid effect on rapidly growing organisms than on bacteria in the stationary growth phase. Bacteria exposed in the cold remove the bactericidal potency of the platelet extract yet are not killed, suggesting that adsorption of the protein onto the negatively charged bacterial surface may not completely explain the bactericidal effect. Basic proteins easily adsorb to negatively charged surfaces, even to the surfaces of artificial particles such as phosphatidylserine vesicles (18). However,

only certain of such adsorbable proteins alter the cation permeability of such particles. Previous studies suggest that basic proteins may act as metabolic inhibitors or may alter membrane permeability. Amano et al. (19) demonstrated that plakin, a water extract of horse platelets, caused marked inhibition of oxygen uptake by susceptible aerobic bacteria. Zeya and Spitznagel (7) showed similar inhibition of oxygen consumption by bacteria exposed to cationic proteins prepared from rabbit leukocyte granules. They demonstrated that the bacteria underwent changes in cell permeability and correlated leakage of nucleotide components with bacterial killing after incubation with leukocyte cationic proteins. The sensitivity of rapidly growing *B. subtilis* to killing by platelet cationic protein, and the similarity of this platelet factor to the cationic proteins of leukocyte granules, strongly suggests that the bactericidal mechanism may similarly involve alterations in permeability and oxidative metabolism.

What is the possible physiological role for a platelet-derived bactericidal substance? Different species of animals vary widely in serum beta lysin activity. Cationic extracts of normal human platelets demonstrate no bactericidal activity. Whether human platelets can develop such activity as part of the inflammatory response has not been determined. Many foreign particles and materials which may gain access to the bloodstream during infection or tissue injury are capable of producing platelet aggregation (20).

Local intravascular aggregation of platelets by appropriate circulating stimuli, such as bacteria, bacterial products such as endotoxin, or antigen-antibody complexes, may initiate the release of bactericidal platelet protein into the blood. Hunder and Jacox demonstrated that rabbits given intraperitoneal injections of endotoxin developed an increased level of serum bactericidal activity (4). Des Pres et al. showed that incubation of rabbit platelet-rich plasma with endotoxin released bactericidal activity (21). Recently, Clawson and White reported the aggregation of platelets by bacteria (22). The release of locally high concentrations of platelet bactericidal protein may permit killing of susceptible bacteria trapped in the platelet aggregates. The question whether subsequent ingestion of platelet-bacterial clumps by polymorphonuclear leukocytes is facilitated by the release of platelet constituents in the aggregates, coating the bacteria, remains to be answered.

These observations suggest that the platelet, like the leukocyte, is capable of participating in the development of the inflammatory response in a variety of ways. Platelet constituents released after interaction of platelets with inflammatory stimuli include nucleotides, vasoactive amines, antiheparin factor, and cationic proteins possessing permeability-enhancing and bactericidal capacity. These factors may be present in relatively high concentrations in the vicinity of the platelet aggregate at the vascular endothelial surface. Their role in affecting or altering that surface remains to be explored.

SUMMARY

The heat-stable antibacterial activity of rabbit serum against Gram-positive microorganisms has been shown to reside in a cationic protein fraction of platelet lysosomal granules. The activity is released during platelet aggregation. No plasma or serum component is required for the bactericidal effect. The platelet bactericidin resembles the antibacterial proteins of leukocyte granules both in cellular localization and in biochemical characteristics. It can be differentiated from platelet factor 4, the antiheparin factor, which is also a basic protein in platelet granules. The antibacterial effect of the platelet bactericidin may be related to the metabolic activity of the organisms. This antibacterial activity of platelets may represent another means by which platelets can participate in host inflammatory defense reactions.

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