INFECTION AND IMMUNITY, Jan, 1974, p. 179-186

Vol. 9, No. 1
Printed in U.S.A.

Release of β-Lysin from Platelets by Thrombin and by a Factor Produced in Heparinized Blood

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Received for publication 30 July 1973

Removal of calcium by either citrate, oxalate, or ethylenediaminetetraacetate inhibited coagulation of blood at an early stage and prevented the release of β-lysin from platelets. In contrast, heparin caused platelet agglutination and stimulated β-lysin release in vitro and in vivo. This release was calcium dependent and may have been due to a calcium-dependent reaction in the blood coagulation sequence. Thrombin which by-passed the early calcium-dependent stages of coagulation directly stimulated the release of β-lysin from platelets. However, thrombin alone or in combination with other plasma factors was not as effective in releasing β-lysin as the regular coagulation process. Thrombin's platelet degranulating activity correlated with its β-lysin releasing activity. In contrast to thrombin, staphylococcal coagulase, which also by-passed the calcium-dependent stages of coagulation, coagulated citrated blood without releasing β-lysin. The release of β-lysin has been observed previously in the absence of blood coagulation, but this is the first observation of coagulation without β-lysin release. It is clear that β-lysin is released from platelets during coagulation by the direct action of thrombin and that it may be released in an earlier calcium-dependent reaction.

In 1901 Gengou (6) reported that, in comparison with serum prepared from whole blood, plasma serum exhibited only low levels of bactericidal activity for gram-positive bacteria (plasma serum was prepared from coagulated plasma which had been depleted of all cells and platelets prior to clot formation). Later, the substance responsible for this activity in serum was named β-lysin. In 1958, Donaldson and Marcus (3) confirmed Gengou's observation in experiments comparing β-lysin activities in plasma and serum. They demonstrated that anticoagulants were not directly responsible for the lack of plasma bactericidal activity, because addition of anticoagulants to serum did not alter its bactericidal effect. In 1960, Hirsch (9) demonstrated that β-lysin activity could be obtained in plasma serum if platelets were restored to the plasma prior to clot formation, thus establishing that blood platelets were the source of β-lysin in blood serum. This was later confirmed by the observation (4) that antisera prepared against purified β-lysin completely neutralized this bactericidal activity in serum and platelet extracts. Furthermore, all of these data show that blood coagulation is essential in the liberation of β-lysin from platelets. However, no attempt has been made to determine which reactions in the coagulation mechanism are responsible for releasing the intracellular β-lysin. The objective of the present study was to determine the β-lysin-releasing reactions in the blood coagulation sequence.

MATERIALS AND METHODS

Animals. New Zealand white rabbits weighing 5 to 6 lb were obtained from a local supplier. They were individually caged, fed rabbit pellets, and given water ad libitum.

β-Lysin assay. β-lysin was assayed as described by Donaldson et al. (2). This procedure requires that a standard inoculum of *Bacillus subtilis* be added to various dilutions of a test sample. After incubation, pour plates were prepared, and the cell viability was determined for each dilution. The reciprocal of the highest dilution which was lethal for a minimum of 99% of the bacteria was the β-lysin titer for the test sample.

β-Lysin neutralization. The anti-β-lysin plasma used in all neutralization tests was prepared in guinea pigs by injecting purified rabbit β-lysin as previously reported (4). Equal volumes of the sample and the anti-β-lysin plasma were mixed and allowed to incubate at 37 C for 30 min. After this neutralization period, bactericidal tests were carried out in the usual manner. It was necessary to use guinea pig anti-β-

lysin plasma rather than serum when neutralizing rabbit plasma samples containing β-lysin. Otherwise the thrombin in guinea pig antiserum would coagulate the plasma, trapping the bacteria in the clot during the β-lysin assay.

Preparation of platelet-rich plasma. Platelet-rich plasma (PRP) was prepared from a freshly drawn blood sample containing 0.38% sodium citrate. This blood was centrifuged at 100 × *g* for 15 min, and the platelet-rich supernatant fluid was carefully aspirated. A model F Coulter counter was used to count the platelets.

Electron microscope examination of platelets. Platelets were sedimented from PRP at 900 × *g* for 10 min. The plasma was poured off, and the platelet button was fixed in a gluteraldehyde-acrolein mixture followed by OsO_4. The platelets were then soaked in uranyl acetate, dehydrated, embedded in plastic, sectioned, stained with lead citrate, and examined in an electron microscope. This procedure is described in detail by Hess (8).

Staphylococcal coagulase preparation. A hospital isolate of *Staphylococcus aureus* was grown from 2 to 5 days in nutrient broth enriched with 0.75% glucose and buffered with 1.0% potassium bicarbonate (15). The bacteria were removed by centrifugation at 29,000 × *g*, and the pH of the culture media was adjusted with HCl to pH 3.8 and allowed to stand at 5 C overnight (16). A coagulase-rich precipitate formed which was collected by centrifugation and resuspended in distilled water, and its pH was adjusted to 7.5. The coagulase was reprecipitated with ammonium sulfate at 45% saturation and collected again by centrifugation. The precipitate was resuspended in a small volume of distilled water and dialyzed overnight against physiological saline solution (PSS). The coagulase was heated to 60 C to destroy any residual α-hemolysin and was sterilized by filtration through a membrane filter (Millipore Corp.). The staphylococcal coagulase preparation was assayed by the procedure described by Tirunarayanan (15), and 8 to 32 coagulase units/ml were used to coagulate PRP.

Thrombin and heparin. Bovine thrombin obtained from K and K Laboratories, Hollywood, Calif., was used in a concentration of 0.66 mg/ml of blood or PRP. The heparin used was an aqueous preparation obtained from Riker Laboratories, Northridge, Calif.

RESULTS

Anticoagulants and β-lysin release. To assess the β-lysin-releasing capacity of early reactions in the blood coagulation sequence, the release of β-lysin was studied in the presence of anticoagulants which block coagulation prior to the generation of thrombin (Table 1). Blood samples were collected from rabbits by cardiac puncture in syringes containing the indicated anticoagulants. The blood samples were incubated for 1 h at 37 C to allow ample time for the coagulation process to proceed to the blocked step. The samples were then centrifuged, and the supernatant fluids were assayed for β-lysin. In the absence of anti-coagulant, the β-lysin titers ranged from 32 to 128 U/ml. In the presence of sodium citrate, potassium oxalate, or ethylenediaminetetraacetate, the β-lysin titers were reduced to 1 or 2 U/ml. This result was anticipated, because all of these agents chelate calcium ions and block the coagulation mechanism at an early step. Heparin, however, only partially blocked the β-lysin release. Sixteen of the 64 β-lysin units present in serum were released in the presence of heparin. Treatment with anti-β-lysin plasma neutralized the bactericidal activity in all cases, indicating that the lethal agent was in fact β-lysin.

In an effort to understand the release of β-lysin that occurred in the presence of heparin, a study comparing the effect of various concentrations of heparin on whole blood was carried out. It was anticipated that the higher concentrations of heparin would be most effective in inhibiting the release of β-lysin, the rate and quantity of β-lysin release increased with heparin concentration (Fig. 1). Either heparin stimulated an early β-lysin releasing step in the coagulation process or it activated a β-lysin-releasing mechanism which was independent of the coagulation process. However, an investigation of the divalent ion requirement of the

TABLE 1. *Effect of anticoagulants on the release of β-lysin from blood platelets*

Anticoagulant	β-lysin (U/ml)	
	No treatment[a]	Anti-β-lysin treatment
None (serum)	64 (32–128)	<2[b]
Sodium citrate (0.38%)	2 (2–8)	<2
Potassium oxalate (0.25%)	2 (1–8)	<2
EDTA (0.2%)	1 (1–2)	<2
Heparin (10 USP U/ml)	16 (16–32)	<2

[a] Values represent median and (in parentheses) range.

[b] After dilution of the test sample with an equal volume of anti-β-lysin, 2 U/ml is the smallest amount of β-lysin that can be measured. In reality, the antibody has probably completely neutralized the bactericidal activity, because no killing of bacteria was evident in any tubes containing anti-β-lysin.

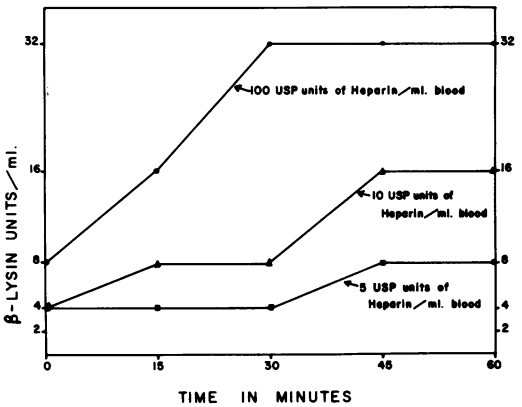

FIG. 1. *Effect of heparin concentration on the release of β-lysin in whole blood.*

heparin-induced β-lysin release revealed that the ion requirements were similar to ion requirements in early steps in the coagulation process (Table 2). The addition of calcium to heparinized whole blood stimulated the release of additional β-lysin, whereas the removal of calcium by the addition of sodium citrate blocked the β-lysin-releasing mechanism. The ionic specificity of this reaction was indicated by the fact that calcium would remove the block, whereas magnesium would not.

A study of the effect of heparin on PRP was carried out to examine the effect of this β-lysin release on the blood platelet. PRP was prepared with citrate, because it was not possible to obtain a preparation with high concentrations of platelets when heparin alone was used as the anticoagulant. Ten USP units of heparin per ml and 3.0×10^{-5} mol of calcium per ml were added to one portion of PRP prepared with citrate. The untreated PRP served as a control. The concentration of calcium used was sufficient to allow the PRP to clot in the absence of heparin. At various times after the calcium and heparin additions, samples were collected, and the extracellular β-lysin level and platelet number were determined (Fig. 2). The addition of calcium and heparin caused a rapid drop in platelet number and a corresponding increase in the β-lysin concentration. In contrast, the controls showed only a slight decrease in platelet number and a correspondingly small increase in β-lysin. At the conclusion of the experiment, an examination of the control platelets under a phase contrast microscope revealed that they were slightly rounded, but otherwise they appeared to be normal (Fig. 3A). In contrast, nearly all treated platelets were found in aggregates like the one in Fig. 3B. This agglutination phenomenon accounts for the large decrease in platelet number, as measured by the Coulter counter (Fig. 2). Individually, the agglutinated platelets looked rounded and deformed. These changes in morphology and the agglutination reaction were dependent on both calcium and heparin, because platelets (Fig. 4) in heparin alone were indistinguishable from the controls. The typical alterations in platelets after a 60-min treatment with heparin and calcium are seen in Fig. 4B. These platelets clumped very tightly together, membrane damage was evident, and most of the platelets appeared to be in the process of breaking up.

In view of the release of heparin and β-lysin (14) during anaphylaxis, a study was initiated

TABLE 2. *Effect of calcium on the release of β-lysin in heparinized whole blood*

Anticoagulant treatment	Divalent ions added	β-lysin (U/ml)[a]
Heparin[b]	None	16 (16–32)
Heparin	Ca^{2+}[c]	32 (16–32)
Heparin + citrate	None	4 (2–4)
Heparin + citrate	Ca^{2+}[c]	32 (16–32)
Heparin + citrate	Mg^{2+}[c]	4 (2–4)

[a] Values represent median and (in parentheses) range.

[b] Heparin concentration was 10 USP U/ml.

[c] Ca^{2+} and Mg^{2+} were adjusted to a final concentration of 3.0×10^{-5} mol/ml.

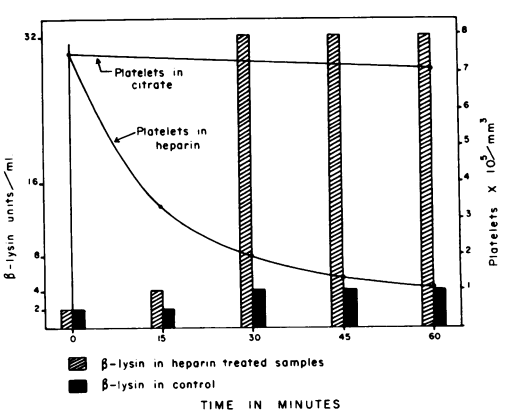

FIG. 2. *β-lysin release and platelet agglutination in heparinized platelet-rich plasma.*

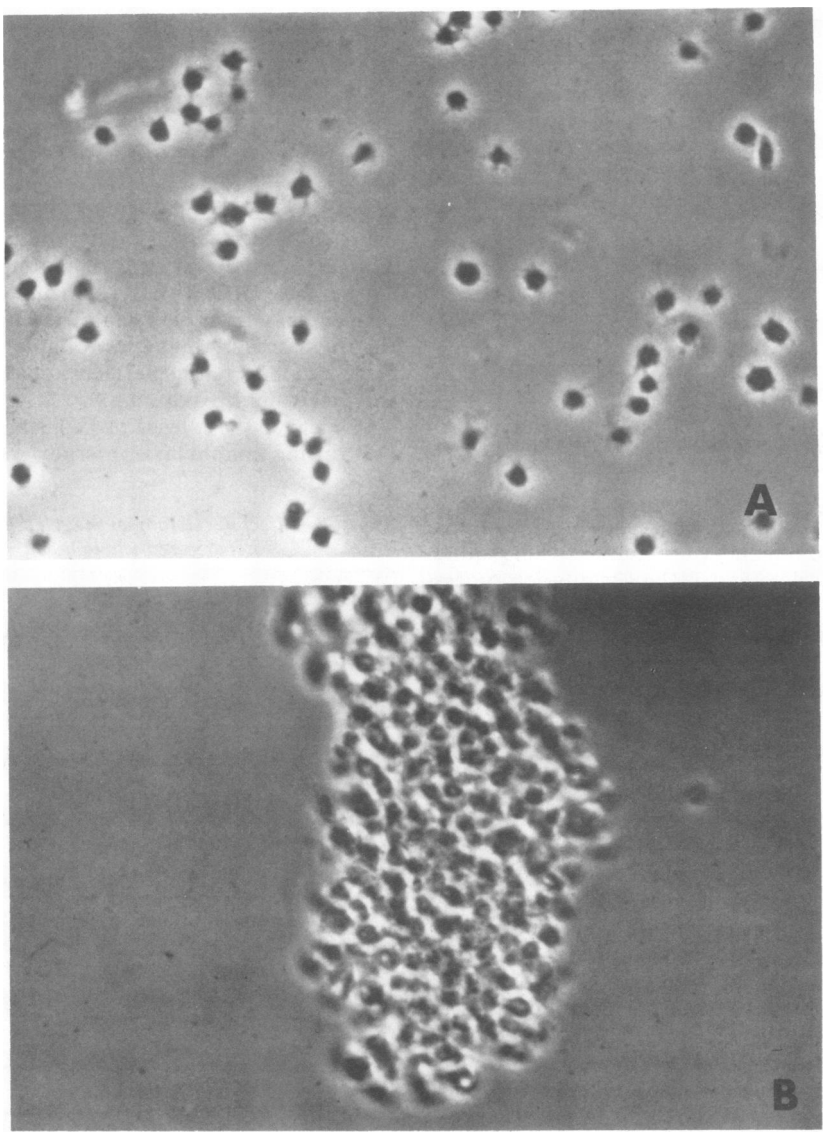

FIG. 3. *Phase-contrast micrographs of platelets after incubation for 1 h in: A, citrate; B, citrate plus heparin and calcium.* ×2,400.

to determine whether heparin would cause the release of β-lysin in vivo. From 5,000 to 20,000 USP units of heparin were injected intravenously into nine rabbits. Two of the nine animals were refractive, and the plasma β-lysin levels were unchanged. The other seven rabbits showed a transient but significant increase in plasma β-lysin. The increase in β-lysin occurred between 5 and 30 min and reached levels four to eight times the initial plasma concentrations. This heparin-induced in vivo release of β-lysin was less dramatic and more transient than that which follows anaphylaxis; consequently, it is doubtful whether the release of heparin could completely explain the β-lysin release during anaphylaxis.

Effect of thrombin and coagulase on extracellular β-lysin. The reactions of the blood coagulation sequence which occurred in the presence of chelating agents or heparin preceded the generation of thrombin. To test the β-lysin-releasing capacity of the next reactions

in the coagulation sequence, thrombin and staphylococcal coagulase were added to citrated blood (Table 3). The addition of calcium served as a positive control by activating the normal blood coagulation mechanism which in turn released serum levels of β-lysin. The addition of thrombin, which by-passed the early calcium-dependent stages of coagulation, coagulated the blood and released β-lysin from the platelets. However, thrombin was only about one-fourth as effective as calcium in releasing β-lysin. In contrast to the obvious release with

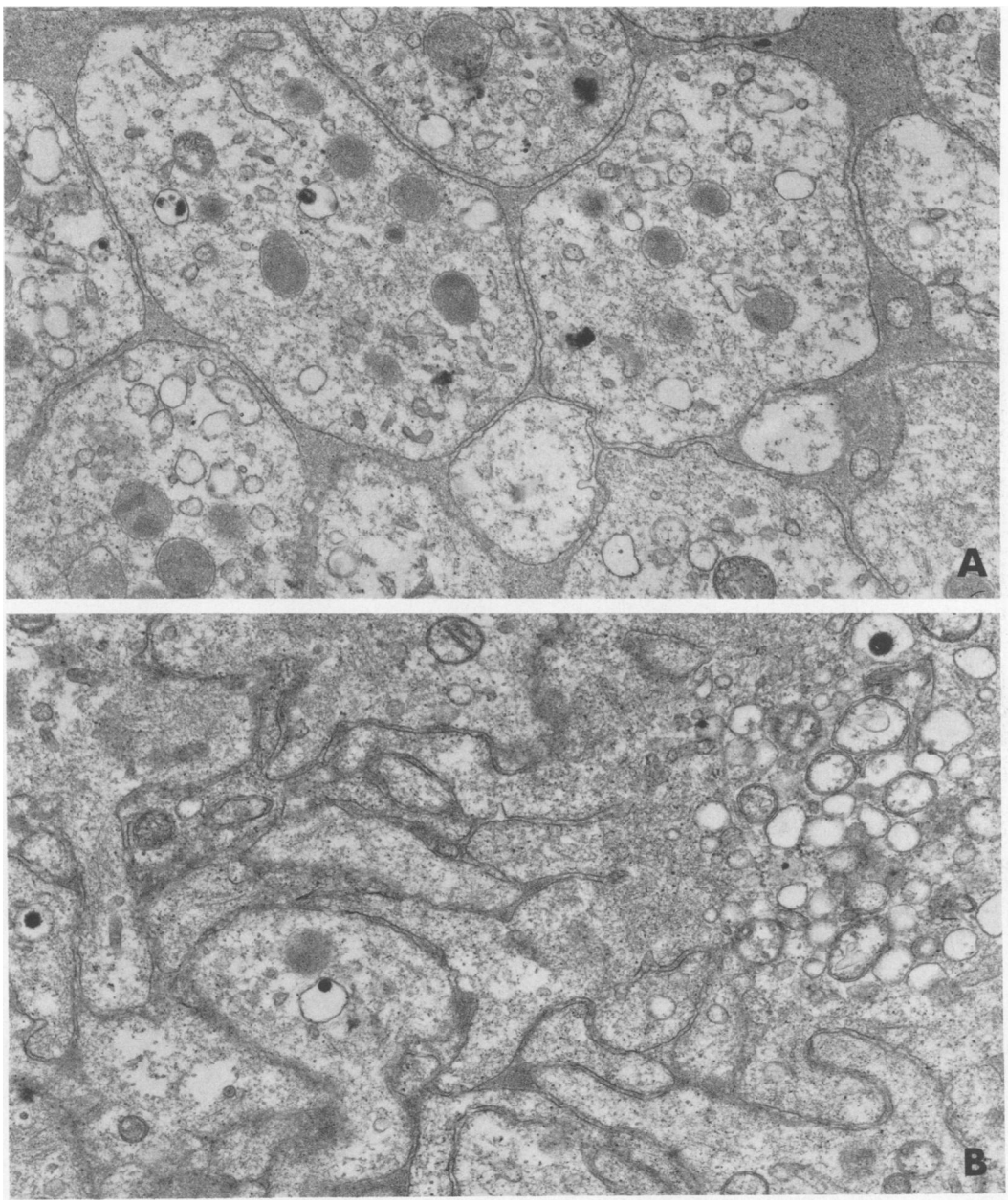

FIG. 4. *Electron micrographs of platelets after incubation for 1 h in: A, citrate; B, citrate plus heparin and calcium.* ×*13,300.*

TABLE 3. *Effect of thrombin and staphylococcal coagulase on the release of β-lysin in citrated whole blood*

Additions	Coagulation	β-lysin (U/ml)[a]
Calcium	Positive	64 (32-64)
Thrombin	Positive	16 (8-64)
Coagulase	Positive	4 (1-4)
None	Negative	2 (2-8)

[a] The median for each group was obtained from a minimum of six animals; values represent median and (in parentheses) range.

TABLE 4. *Effect of thrombin on the release of β-lysin from platelets*[a]

Sample	β-lysin (U/ml)	Increase over platelets in PSS[b]
Platelets in PSS	2 (1-4)	
Platelets in PSS + thrombin	8 (8-16)	4× (2×-16×)
Platelets in plasma + thrombin	32 (32-128)	16× (8×-128×)
Plasma alone	2 (1-2)	None

[a] Values represent median and (in parentheses) range.

[b] The median was determined from values obtained from six animals. PSS, Physiological saline solution.

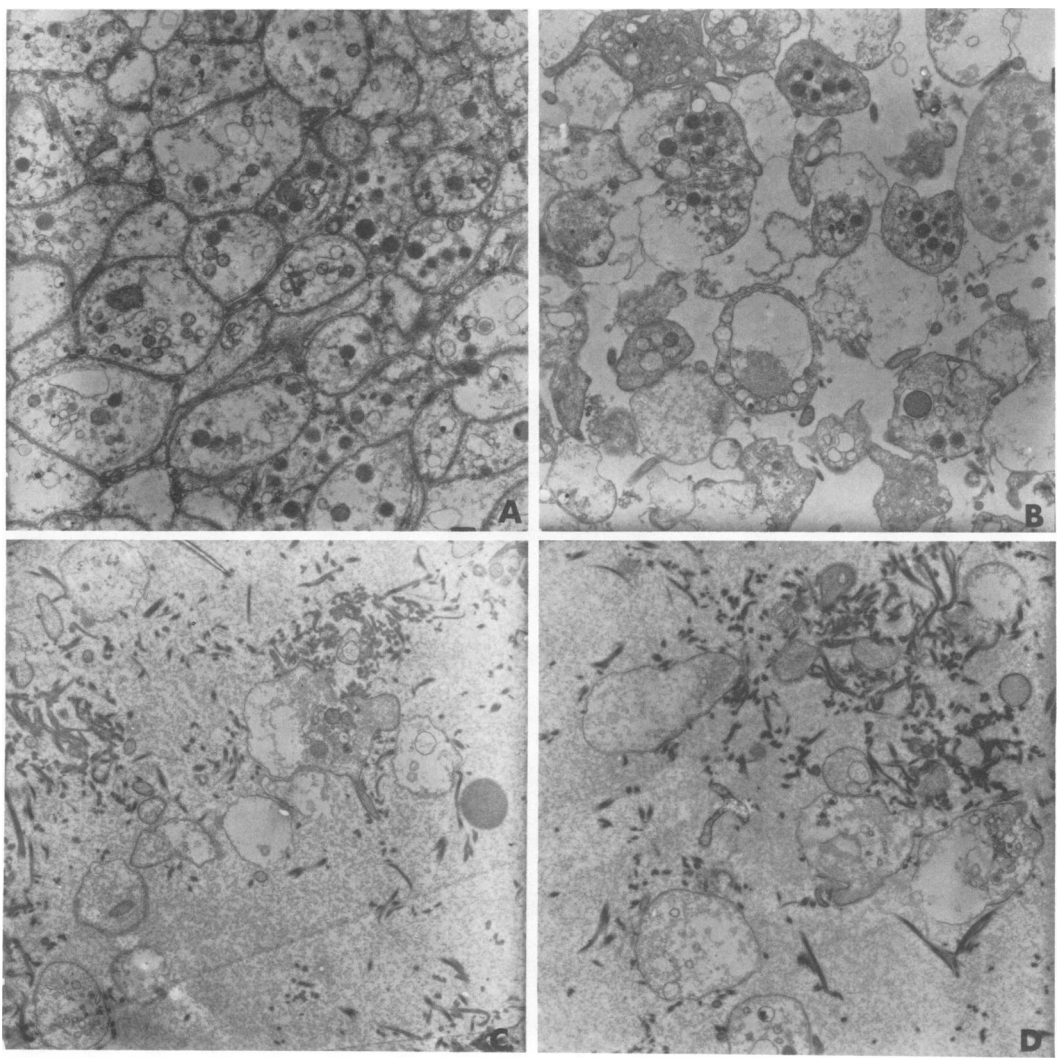

FIG. 5. *Effect of thrombin on rabbit platelets. A, Control platelets in PSS; B, platelets in PSS plus thrombin; C and D, platelets in citrated plasma plus thrombin. All platelets were from the same rabbit.* ×4,700.

thrombin, the addition of staphylococcal coagulase, which also by-passed the calcium-dependent stages of blood coagulation, coagulated the citrated blood without releasing significantly more β-lysin than the untreated control. Coagulase was not directly neutralizing β-lysin, because the addition of coagulase to serum did not inhibit bactericidal activity. It should be emphasized that in this experiment the coagulase preparation cannot be contaminated with staphylococcal α-hemolysin, because α-hemolysin will lyse platelets and release β-lysin. The inability of staphylococcal coagulase to release β-lysin may be related to the clot retraction reaction. Whenever the fibrinogen-to-fibrin reaction was driven by thrombin, good clot retraction was observed. Staphylococcal coagulase caused coagulation with little or no clot retraction.

To determine whether the thrombin-mediated β-lysin release was due to the direct action of thrombin or to a subsequent reaction, thrombin was added to a platelet preparation in the absence of the plasma proteins (Table 4). In this study, PRP was separated from citrated whole blood, and the platelets were sedimented at 900 × *g* for 5 min. The platelet buttons were then washed twice in PSS to remove any residual plasma proteins and resuspended in either PSS or plasma. The addition of thrombin to platelets in PSS alone resulted in a fourfold increase in extracellular β-lysin, indicating that thrombin will act directly on the platelet to release β-lysin. However, the addition of thrombin to platelets in plasma resulted in a 16-fold increase in extracellular β-lysin, indicating that a reaction subsequent to thrombin activation must play a role in β-lysin release or that a plasma factor must normally assist thrombin in releasing β-lysin from platelets. Examination of these platelets under an electron microscope revealed that the addition of thrombin induced extensive platelet degranulation. Figure 5 shows the typical alterations observed in thrombin-treated rabbit platelets. The β-lysin results obtained from this particular rabbit were as follows: platelets in PSS released 1 U of β-lysin, platelets in PSS plus thrombin released 16 U of β-lysin, and platelets in plasma plus thrombin released 32 U of β-lysin. The PSS control platelets in Fig. 5A show evidence of damage and a loss of internal material. This damage may have been due to the repeated washings and the long period of standing in PSS. However, in comparison with platelets treated with thrombin in either PSS or plasma, the untreated platelets are basically intact. The addition of plasma to thrombin caused extensive platelet degranulation. The extent of platelet damage correlated with the release of β-lysin.

DISCUSSION

The intrinsic system of blood coagulation is presented in Fig. 6 (1, 10). The first two reactions in this system (numbers XII and XI) could not have been responsible for β-lysin release, because they are independent of calcium and proceed in the presence of the chelating anticoagulants which block β-lysin release. Likewise, the fibrinogen-to-fibrin reaction may be eliminated as a β-lysin-releasing reaction, because staphylococcal coagulase can drive this reaction in the presence of platelets and not release β-lysin. Heparin has some inhibitory activity on several steps in the coagulation process (7); however, its major activity is to prevent the conversion of prothrombin to thrombin (11, 13). Thus in the presence of this anticoagulant, three additional reactions, all of which require calcium, could occur, and one of these reactions could be the basis for the heparin-induced β-lysin release. It is possible that β-lysin is released with platelet factors 1 and 3 which are active in these reactions.

Recently Eika (5) reported that heparin increased platelet adhesiveness by causing platelets to release adenosine diphosphate (ADP) which aggregated the platelets. However, when we aggregated platelets in PRP with ADP, we were never able to demonstrate a release of β-lysin, indicating that aggregation and β-lysin release are independent phenomena. Conse-

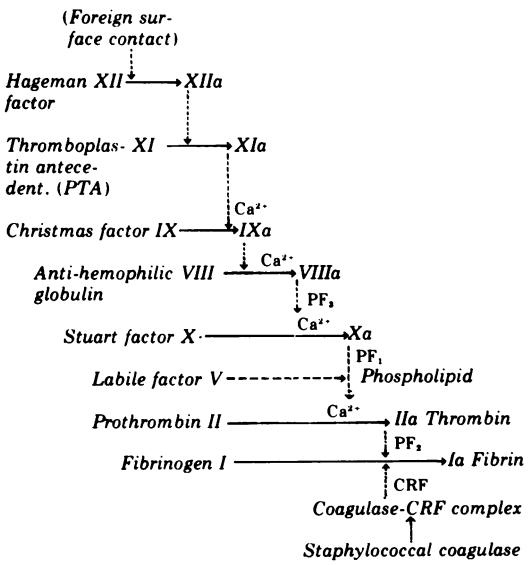

FIG. 6. *The blood coagulation sequence by the intrinsic system and staphylococcal coagulase. PF, Platelet factor; labile factor, proaccelerin, Ac-globulin, prothrombin accelerator; CRF, coagulase reactive factor.*

quently, the release of β-lysin by heparin could not be due to ADP unless ADP induced a second reaction which was calcium dependent. It seems more reasonable to relate the calcium-dependent release of β-lysin to coagulation reactions which are not inhibited by heparin. If this is the case, β-lysin release occurs as a result of at least two coagulation reactions and does not require the entire process.

Although thrombin alone is capable of releasing β-lysin from platelets, its releasing activity is enhanced by other plasma factors. However, even in the presence of these plasma factors, normal coagulation is more effective than thrombin in releasing β-lysin. This also supports the hypothesis that at least two reactions are responsible for the release of β-lysin during normal blood coagulation. The first of these reactions would be calcium dependent, and the second would be the result of thrombin activation.

The finding that staphylococcal coagulase does not release β-lysin is consistent with the recent reports that coagulase does not affect platelets and that its coagulating activity is not mediated by thrombin activation (12). β-Lysin release from platelets into plasma has been previously observed in the absence of blood coagulation (14), but this is the first observation of blood coagulation without a β-lysin release.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-01171 from the National Institute of Allergy and Infectious Diseases.

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