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Beta-Lysin of Platelet Origin

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INTRODUCTION

β-Lysin is the name that Pettersson proposed to distinguish this thermostabile bactericidal component of serum from Buchner's alexin or α-lysin (51). He defined β-lysin as the bactericidal substance found in normal serum that resisted inactivation when heated to 56°C for 30 min. According to this definition, there are at least three different β-lysins found in normal serum; these consist of lysozyme, β-lysin from platelets, and β-lysin of nonplatelet origin. The first of these, lysozyme, is a single entity which is well defined in regard to its chemical and biological properties. The other two types of β-lysins are not readily distinguished from each other, and each could be composed of several distinct molecules. The β-lysin of platelet origin may be similar or identical to plakin (23). The β-lysin of nonplatelet origin may include the bactericidal cationic proteins isolated from leukocyte lysosomes (64–66). In this review, attention will be focused on the β-lysin of platelet origin, which will be referred to as "platelet β-lysin."

BACKGROUND

Most of the history of serum bactericidins, including β-lysin, has been reviewed by Myrvik (46) and Skarnes and Watson (58). Other than reviewing a few historical milestones, which help define platelet β-lysin (Table 1), the history will not be presented here. Although earlier workers had found that plasma was less bactericidal than serum (Gengou, cited in reference 26) and the bactericidal activity of heated serum was much greater for gram-positive bacteria than for gram-negative bacteria (Pirenne, cited in reference 58), Pettersson's original definition of β-lysin did not indicate the source of β-lysin or the types of bacteria killed by β-lysin.

Platelets had been shown to contain an anthracidal agent by Gruber and Futaki in 1907 (23). They used inactivated horse serum to extract a substance from rabbit and horse platelets that they named "plakin." This agent was shown to be active against other members of the *Bacillus* genus by Amano et al. (5, 6). Hirsch (26) related the platelet bactericidal activity to serum bactericidal activity when he showed that platelets had to be present in plasma during coagulation for serum to reach full bactericidal capacity for *Bacillus subtilis*. Attempts to purify the bactericidal component against *Bacillus* from serum had been unsuccessful until the chance observation was made in our laboratory that sterilization of serum by Seitz filtration removed bactericidal activity (10; D. M. Donaldson and B. Ellsworth, Abstr. Int. Congr. Microbiol., 8th, p. 107, 1962). The Seitz filter pad could be washed, and the β-lysin could then be eluted by a high-salt solution. This β-lysin was further purified with *O*-(carboxymethyl)-cellulose chromatography (34). Purified β-lysin prepared from rabbit and rat sera by the Seitz filtration technique was used to prepare an anti-β-lysin in guinea pigs. This anti-β-lysin completely neutralized the bactericidal activity against *B. subtilis* of plakin, purified β-lysin, serum, and aqueous extracts of platelets (11). The anti-β-lysin had no effect on the bactericidal activity of lysozyme, leukins, or aqueous extracts of leukocytes (11, 14). The purification and serological identification have facilitated further characterization of platelet β-lysin.

TABLE 1. *Historical discoveries identifying platelet β-lysin*

Principal contributor	Approximate date	Discovery
J. Fodor	1887	Anthracidal activity of defibrinated blood
E. von Behring	1888	Thermostability of this serum bactericidin
O. Gengou	1901	Release of thermostable bactericidins during blood coagulation
Y. Pirenne	1904	Lethal activity of thermostable bactericidins restricted to gram-positive organisms
M. Gruber	1907	Anthracidal activity of platelet extracts; named active substance plakin
A. Pettersson	1924–36	Named thermostable serum bactericidins, β-lysin; described species distribution and microbial spectrum
J. G. Hirsch	1960	Platelet origin of a serum bactericidin released during blood coagulation
D. M. Donaldson	1961–64	Purification of β-lysin from serum and serological identity of platelet β-lysin and plakin

BIOLOGICAL CHARACTERISTICS OF PLATELET β-LYSIN

The most significant characteristic of platelet β-lysin is its ability to kill a variety of gram-positive bacteria and, as far as is now known, that is its only function. Unlike antibody, platelet β-lysin lacks specificity; its concentration in serum is not increased by immunization with a susceptible organism (10, 48). Since germfree rats have fully as much platelet β-lysin as conventional rats (28), exposure to viable microorganisms in the environment does not appear to increase serum levels.

The bactericidal activity of platelet β-lysin forms the basis for the methods used to study this substance and to analyze its role in vivo. Two similar assay systems have been most frequently used to quantitate β-lysin concentrations in serum and other fluids. The first method (10, 26) determines the number of organisms killed as determined by a plate count procedure; the second is a more rapid photometric assay, which, unfortunately, does not distinguish between bactericidal and bacteriostatic activity. The first assay procedure, as used in our laboratory (10), requires that a standard inoculum of *B. subtilis* (approximately 30,000 viable bacteria/ml) be added to various dilutions of a test sample. Under these assay conditions, the bacteria are killed rapidly. Over 80% of the standard inoculum is killed within 2 min (40), and maximum platelet β-lysin titers are obtained between 15 and 40 min (unpublished observation). To determine the extent of killing during a standard incubation period of 1 to 2 h, pour plates are prepared from each dilution and incubated overnight. The next day the colonies on each plate are counted, and the number of viable bacteria remaining in each dilution after incubation is determined. A unit of β-lysin is defined as the amount of β-lysin required to kill 99% of the original bacterial inoculum. The β-lysin titer for a test sample would then be the reciprocal of the highest sample dilution that was lethal for a minimum of 99% of the bacteria. Since a number of bactericidal agents other than platelet β-lysin will kill *B. subtilis*, it is important to determine whether a lethal effect is due to platelet β-lysin or to another bactericidal agent. This is most easily accomplished by using a specific antibody (anti-β-lysin) that will specifically neutralize the bactericidal activity of platelet β-lysin (11). Under these conditions, the bactericidal activity of a test sample can only be attributed to platelet β-lysin if the lethal activity can be neutralized by exposure to anti-β-lysin.

Through the use of the above assay system, the serum platelet β-lysin concentration has been determined in a variety of mammalian species. In descending order, typical serum concentrations as determined in our laboratory were 64 to 512 U/ml in rats, 16 to 64 U/ml in rabbits, 2 to 8 U/ml in horses, 1 to 4 U/ml in humans, and 1 to 2 U/ml in cows (28, 33, 42). The sera of sheep, cats, swine, guinea pigs, and mice have also been assayed for platelet β-lysin, but the level was either too low to measure or completely absent. Platelet β-lysin has not been found in the serum of any animal division other than mammals, and there is no evidence for its existence in any plant or microorganism. Serological studies of platelet β-lysin in different species have shown that platelet β-lysin from rats, humans, and horses can be neutralized by an anti-rabbit β-lysin made in guinea pigs (42). However, this antibody was more effective in neutralizing rabbit platelet β-lysin than rat or human platelet β-lysin (42). These results indicate that platelet β-lysins from different species resemble one another but that differences do exist.

In addition to serum, platelet β-lysin has been found in rabbit plasma, saliva, and aqueous humor (33). However, the concentration in these body fluids was only 1 to 4 U/ml as opposed to 16 to 64 U/ml in serum. In a recent study by Ford et al. (18), high levels of platelet β-lysin, ranging from 32 to 64 U/ml, were found in human tears. This is particularly remarkable in view of the low levels in human serum. In the same study human aqueous humor, like rabbit aqueous humor, contained 1 to 4 U of platelet β-lysin per ml. Recently, Martinez (39) reported the existence of heat-stable components in human lymph that killed *B. subtilis*. At least two bactericidal agents appear to be present in lymph (R. J. Martinez, personal communication). The major killing agent has been identified as lysozyme. The other bactericidal agent(s) has not been identified, but could be platelet β-lysin, since it is a heat-stable cationic compound. Platelet β-lysin has never been found in normal vitreous humor, spinal fluid, urine, or washes of normal tissues (33).

The first clue that platelets might be the cellular source of serum β-lysin came in 1901 when Gengou (cited in reference 26) reported that the blood coagulation process was of significance in the release of β-lysin. Gengou's observation was later confirmed, and it was demonstrated that anticoagulants were not directly responsible for the lack of β-lysin activity in plasma, since the addition of anticoagulants to serum did not alter its bactericidal activity (12). In 1960 Hirsch (26) prepared plasma without anticoagulants by use of low temperatures and siliconized glassware. He observed that serum obtained from such plasma manifested essentially no bactericidal activity. However, when he added platelets to such plasma before coagulation, serum bactericidal activity was restored. Later, it was reported that antisera prepared in guinea pigs against purified rabbit β-lysin neutralized the bactericidal activity of serum, platelet extracts, and purified β-lysin (11). The above studies indicated that the origin of β-lysin was the blood platelet, and that β-lysin was released from platelets during the blood coagulation process. Studies to determine where the bactericidal activity for *B. subtilis* was stored in the platelet indicated that the storage organelle was the lysosomal granule (63). All attempts to find platelet β-lysin in other cells, especially macrophages and neutrophils, have been unsuccessful (14, 33). However, the fact that platelet β-lysin was found in normal body fluids like saliva, aqueous humor, and tears indicates that some platelet β-lysin may come from a source other than the blood platelet. In fact, there may be special cells in the salivary glands and tear ducts that synthesize and secrete platelet β-lysin continuously.

It is believed that platelet β-lysin exerts its lethal effect on susceptible bacteria by disrupting their cell membranes. This was substantiated by the observations that platelet β-lysin had no effect on bacterial cell walls (41), but would combine directly with purified bacterial cell membranes (22) and would lyse bacterial protoplasts (41). In our laboratory, organisms belonging to the genera *Bacillus, Clostridium, Micrococcus, Lactobacillus*, and *Arthrobacter* were susceptible to the action of purified platelet β-lysin. We found that organisms belonging to the genus *Staphylococcus* were not susceptible to the action of the purified platelet β-lysin but were susceptible to nonplatelet β-lysin (34). Recently, Shultz (56, 57) demonstrated that *Listeria* were also susceptible to nonplatelet β-lysin but not to platelet β-lysin. In our studies, the only gram-positive group that was uniformly not susceptible to either platelet or nonplatelet β-lysin was the genus *Streptococcus*. The gram-positive organisms were never killed by the antibody-complement system, and we could not find a single strain of gram-negative organisms that was susceptible to purified platelet or nonplatelet β-lysin. In addition to the gram-negative bacteria, molds, yeasts, viruses, mammalian cells in tissue culture, and mycoplasma were not susceptible to the lethal action of platelet and nonplatelet β-lysin (unpublished observations).

The most important biological characteristics of platelet β-lysin are summarized in Table 2. Many of these characteristics will be discussed in a different context in later sections of this review.

PHYSICAL CHARACTERISTICS OF PLATELET β-LYSIN

The purification of platelet β-lysin made it possible to determine some of its physical characteristics and to substantiate previous findings with whole serum. The Seitz filtration

TABLE 2. *Biological characteristics of platelet β-lysin*

Property	Chartacteristic
Species distribution	Found in variety of mammalian species including rats, rabbits, humans, and cows
Present in body fluids	Serum, plasma, tears, aqueous humor, and saliva
Cellular origin	Blood platelet or thrombocytes
Antigenicity	Neutralizing anti-β-lysin can be produced in guinea pigs against rat and rabbit β-lysin
Principal mechanisms of release	Blood coagulation
Microbial spectrum	Gram-positive bacteria including the genera *Bacillus, Clostridium, Lactobacillus, Arthrobacter*, and *Micrococcus*
Site of action	Cell membrane
Kinetics of reaction	Killing occurs within minutes and is complete within 1 h under standard assay conditions

technique for purifying platelet β-lysin (10; Donaldson and Ellsworth, Abstr. Int. Congr. Microbiol., 8th, p. 107, 1962) consisted of slowly passing normal serum (100 to 150 ml) through either a clarifying or a sterilizing Seitz filter pad. The filtrate contained almost all of the serum protein but had little or no measurable bactericidal activity. The asbestos-cellulose filter pad containing absorbed β-lysin was extensively washed with either water or physiological saline solution. The platelet β-lysin was then eluted with a small volume (15 to 20 ml) of 1.5 M NaCl. This relatively simple technique separated β-lysin from serum lysozyme (14) complement and antibody (10). It yields platelet β-lysin preparations that have specific activities greater than 100 times that of normal serum (10). These platelet β-lysin preparations need to be either diluted or dialyzed before they are tested for bactericidal activity because NaCl concentrations three times physiological (0.45 M NaCl) completely inhibit the bactericidal activity. The Seitz filter eluates could be further purified by *O*-(carboxymethyl)-cellulose chromatography (34). Even with this high degree of purification (24,000 times that of serum for the platelet β-lysin against *B. subtilis* and 37,000 times for the nonplatelet β-lysin against *S. aureus*), the two β-lysins were not completely separated from each other.

Platelet β-lysin is a highly reactive small cationic protein with a molecular weight of approximately 6,000 (Table 3). Purified preparations of platelet β-lysin prepared by the Seitz filtration technique had an ultraviolet light absorption spectrum typical of proteins when examined with a scanning spectrophotometer. These preparations yielded positive protein reactions with the Lowry and biuret methods and negative reactions when tested for carbohydrate or nucleic acid with the Molisch and phenylhydrazine methods (unpublished observations). The bactericidal activity of purified platelet β-lysin was destroyed by the proteolytic enzymes trypsin, pepsin, papain, protease, and fibrinolysin (54). Bactericidal activity was unaltered by the enzymes deoxyribonuclease, ribonuclease, neuraminidase, hyaluronidase, lipase, and phosphatase. Platelet β-lysin was

TABLE 3. *Physical characteristics of platelet β-lysin*

Property	Characteristic
Chemical nature	Cationic protein
Molecular weight	Approximately 6,000
Thermostability	No loss of activity at 95°C for 15 min in purified form
Stability on storage	Stabile in serum at −60°C; unstable in purified form unless lyophilized

concluded to be basic because its bactericidal activity was inhibited by deoxyribonucleic acid, ribonucleic acid (46), and acidic phospholipids (36). Also, platelet β-lysin attaches to Seitz filter pads (10), filter papers, asbestos, bentonite (14), *O*-(carboxymethyl)-cellulose (34), and purified membranes of *Bacillus megaterium* (22). A strong cationic charge could account for the above findings. The removal of platelet β-lysin from solution by these different agents created problems, but was useful in certain studies. An obvious benefit was the use of Seitz filtration as the first step in platelet β-lysin purification. The fact that bentonite removed both platelet β-lysin (14) and lysozyme from serum without altering antibody or complement concentrations (19, 29, 62) allowed the study of the antibody-complement system in the absence of these bactericidins. The type of problems created by the high reactivity of platelet β-lysin is illustrated by the following examples. An assay for platelet β-lysin was developed in which the bactericidal test was carried out in plastic Limbro plates. This test worked well with serum but gave negative results when tested with purified platelet β-lysin. Subsequent work revealed that the purified platelet β-lysin absorbed to the plastic and was inactivated. When purified β-lysin was placed on a Sephadex column in the absence of other proteins, it attached to the beads. It would not pass through the column unless the ionic strength of the eluting fluid was about 10 times the physiological concentration. For this reason, it was necessary to use a 1.5 M NaCl solution in the Sephadex columns used to estimate the molecular weight of the purified platelet β-lysin (34). In normal serum, platelet β-lysin appeared to be attached to other serum proteins, since it was distributed in all protein fractions after either ammonium sulfate precipitation (unpublished observation) or Sephadex gel filtration of serum (14). This may also be a result of its highly reactive cationic nature.

The early work in thermostability of platelet β-lysin was carried out with serum and indicated that bactericidal activity was destroyed at 65 to 68°C for 30 min (51–53). At these temperatures, serum turbidity was increased, indicating that some coagulation was taking place. Purified preparations of platelet β-lysin show no loss of activity under the above conditions, or at temperatures as high as 95°C for 15 min. Bactericidal activity was partially lost at 95°C for 30 min (unpublished observation).

In contrast to the increase in thermostability that accompanied purification, the stability during storage was decreased with purified platelet β-lysin. Serum retained full bactericidal activity for longer than a year if stored at −70°C, but under identical conditions purified platelet β-lysin lost activity within a few days. We found that the most efficient procedure for storing purified β-lysin for long periods of time was in the lyophilized form in the refrigerator (13, 43). The bactericidal activity was reduced by about one-half by the lyophilization procedure, but it could be stored for months without further loss of activity.

There is some confusion as to whether platelet β-lysin is composed of one or two components that are necessary for its bactericidal activity against *B. subtilis*. Myrvik and Leake (47) used ethanol fractionation to separate two non-dialyzable components of rabbit serum that were inactive by themselves but were active in combination with each other. Hunder and Jacox (27) derived two similar protein components from rabbit platelets. The species distribution of the serum components and the platelet components was remarkably similar. Both calcium and bicarbonate ions were required for the bactericidal activity of human serum for *B. subtilis*, whereas only bicarbonate was required for rabbit serum activity (30–32, 48). The major objection to the necessity of a two-component system or an ion requirement for bactericidal activity is that even with serum β-lysin purified 27,000-fold, other serum components are not required for bactericidal activity against *B. subtilis* (34). Further evidence for the lack of ion requirements was that the addition of the chelating agents citrate, oxalate, or ethylenediaminetetraacetate to rabbit serum did not decrease the bactericidal activity of rabbit serum for *B. subtilis* (12, 34). The observation that the bactericidal activity of the purified platelet β-lysin was often increased by the addition of non-bactericidal guinea pig serum (42) indicates that other serum components may increase β-lysin activity, even though platelet β-lysin itself is capable of killing.

DIFFERENTIATION OF β-LYSIN FROM OTHER BACTERICIDAL SYSTEMS FOUND IN NORMAL SERUM

One of the problems in studying serum bactericidins is to determine which bactericidin is responsible for a given bactericidal effect. The distinguishing characteristics of the five major systems found in normal serum are listed in Table 4. Platelet β-lysin is easily distinguished from the antibody-complement and the properdin systems on the bases of its thermostability and the differences in its bactericidal spectrum. However, platelet β-lysin and lysozyme are similar in these respects and more difficult to distinguish. Nevertheless, they differed in a number of respects. (i) Anti-β-lysin serum neutralized the bactericidal activity of platelet β-lysin but not that of lysozyme (14). (ii) Unlike lysozyme, purified platelet β-lysin did not rapidly reduce the optical density of a suspension of susceptible bacteria (14). This is the case because the mechanism of action of lysozyme is on the cell wall, whereas the action of platelet β-lysin is on the cell membrane. As long as the cell wall remains intact, the bacteria will not lyse and the optical density will not be reduced. (iii) Platelet β-lysin and lysozyme were separated by gel filtration. This separation technique works effectively because lysozyme is almost three times as large as β-lysin (≈15,000 as opposed to ≈6,000) (34, 35). (iv) Platelet β-lysin was also separated from lysozyme by the Seitz filtration purification procedure. The platelet β-lysin that was eluted from the Seitz filter with 1.5 M saline was free from lysozyme (14). (v) Platelet β-lysin originates in blood platelets where lysozyme is absent, and blood leukocytes are rich in lysozyme but have no platelet β-lysin (14, 33).

Platelet β-lysin and the nonplatelet β-lysin, which will kill *S. aureus*, were difficult to distinguish (34). They were both found to be cationic proteins of approximately 6,000 molecular weight. Their absorption and elution properties on cellulose asbestos filters and on *O*-(carboxymethyl)-cellulose columns were similar. Both were released during blood coagulation, and both were resistant to inactivation upon heating at 95°C for 5 min. However, they differ in a number of respects (34): (i) non-platelet β-lysin would kill *S. aureus*, whereas platelet β-lysin would not; (ii) platelet extracts did not contain nonplatelet β-lysin; (iii) nonplatelet β-lysin was inactive in the presence of heparin, sodium citrate, sodium oxalate, ethylenediaminetetraacetic acid, acid phospholipid, and acid pH, whereas platelet β-lysin was active under these conditions; (iv) nonplatelet β-lysin has only been found in rabbit and horse sera, whereas platelet β-lysin has been found in the sera of other species and was detected in especially high levels in rat serum; (v) a variety of proteins, including normal guinea pig serum, inhibited the bactericidal activity of the nonplatelet β-lysin for *S. aureus*. In contrast, normal guinea pig serum and other proteins did not diminish the activity of platelet β-lysin (11). This nonspecific inactivation of nonplatelet β-lysin makes serological studies difficult. It is not known at this time whether the nonplatelet β-lysin that kills *Listeria monocytogenes* (56, 57) is the same as the one that kills *S. aureus* (34). They appear very similar, although the molecular weight, most of the ion effects, and

TABLE 4. *Comparison of bactricidal systems found in normal serum*

System	Thermostability (56°C, 30 min)	Typical test organism	Bactericidal spectrum	Serum concn vs. plasma concn	Origin	Chemical nature	Mol wt	Site of action	Mode of action
Platelet β-lysin	Stable	*B. subtilis*	Gram positive	Serum ≫ plasma	Platelets, lysosomes	Basic protein	6,000	Cell membrane	Non-enzymatic (similar to other cationic proteins)
Nonplatelet β-lysin	Stable	*S. aureus* or *L. monocytogenes*	Gram positive	Serum > plasma	?	Basic protein	6,000	Cell membrane for *Listeria*	?
Lysozyme	Stable	*M. lysodeikticus*	Gram positive	Serum = plasma	Leukocytes, lysosomes	Basic protein	15,000	Cell wall	Enzymatic reaction
Antibody (Ab) complement (C′)	Labile	*E. coli*	Gram negative	Serum = plasma	Plasma cells (Ab) Intestinal and liver cells (C′)	Proteins and glycoproteins	150,000–900,000 (Ab) 70,000–400,000 (C′)	Cell wall and cell membrane	Antibody binding and cascading enzymatic reactions
Properdin	Labile	*E. coli*	Gram negative	Serum = plasma	?	Proteins and glycoproteins	23,000–223,000	Cell wall and cell membrane	Cascading enzymatic reactions

the protein effects have not been determined using the nonplatelet β-lysin for *L. monocytogenes*.

It appears likely that platelet β-lysin and plakin are identical substances. This hypothesis is supported by the observations that both anti-β-lysin serum (11) prepared against purified β-lysin and anti-platelet serum prepared against whole platelets (27) caused complete neutralization of bactericidal activity against *B. subtilis* in whole serum and in crude plakin preparations. In a series of papers through the 1950s and 1960s, Amano and co-workers characterized plakin (1-8, 24, 25). The hypothesis that platelet β-lysin and plakin are identical substances was supported by most of their work. In addition to the observation that plakin was present in rat, rabbit, and horse platelets, as was reported by Gruber and Futaki (23), the following characteristics of plakin, as reported by Amano's group, are consistent with the known characteristics of platelet β-lysin. (i) plakin was bactericidal against *Bacillus anthracis*, *B. megaterium*, and *B. subtilis* but not against *S. aureus* (5-7); (ii) plakin had no effect on bacterial cell walls but acted on the cell membrane and lysed *B. megaterium* protoplasts in sucrose medium (4); (iii) plakin was found to be stable at 60°C for 1 h at pH 7.0 (7); (iv) chemically, plakin was found to be a protein; (v) plakin was absorbed very quickly by bacterial cells; (vi) the activity of plakin was lost within a few days upon storage in a refrigerator (24). However, the following characteristics of plakin are at variance with the known properties of β-lysin: (i) a crude plakin extract was not inhibited by ribonucleic acid and, in this respect, differed from basic polypeptides, protamine, and lysozyme (5, 7); (ii) the divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+} were found to be indispensible for the activity of plakin (37).

In our experience it was more difficult to get ribonucleic acid to neutralize platelet β-lysin than many other anionic substances. The reason for this is not clear, but it may relate to the presence of small amounts of ribonuclease, which could destroy the ribonucleic acid. We were unable to demonstrate any divalent cation requirements for platelet β-lysin using high concentrations of ethylenediaminetetraacetic acid (approximately the same concentration as Amano's group) and a variety of other divalent cation chelating agents (12, 60). However, our assay for platelet β-lysin and Amano's assay for plakin are very different. In contrast to the bactericidal assay used in our laboratory, Amano's group measured changes in oxygen uptake by a manometric technique (7). A decrease in oxygen consumption by sensitive bacteria was an indication of bactericidal activity in their system. They use this assay because it was rapid and they did not purify plakin aseptically (7). Perhaps the difference in ion requirements relates to the difference in assay procedure. Amano's group also believes that plakin is a phospholipase, because they can demonstrate phospholipase activity in a partially purified plakin preparation (25). Purified platelet β-lysin has never been tested under identical conditions, but attempts in our laboratory to demonstrate phospholipase activity on purified bacterial membranes were unsuccessful. In spite of these apparent differences, we still suspect that platelet β-lysin and plakin are the same substance.

RELEASE OF β-LYSIN FROM PLATELETS

The amount of platelet β-lysin in plasma is low when compared to the amount potentially available in thrombocytes (33). However, platelet β-lysin would be effective as a humoral bactericidal agent if it were released in an active form during infection or when the potential for bacterial infection was great. In this context a number of biological processes associated with the infectious process itself or that would occur when the potential for infection was high were studied. The combined results from a number of studies that indicate that platelet β-lysin is released under such circumstances are summarized in Table 5.

Blood coagulation and the associated release of platelet β-lysin has generally been studied in vitro. However, it is clear that wounds in the body caused by incisions, lacerations, or punctures generally disrupt the blood vascular system and stimulate the blood coagulation se-

TABLE 5. *Biological processes that mediate the release of β-lysin from platelets*

Process	Increase in free platelet β-lysin over that of normal plasma
Blood coagulation	8- to 64-fold
Inflammation	8- to 32-fold (the specific activities of inflammatory exudates were generally over 100-fold greater than the specific activities obtained in normal plasma)
Antigen-antibody reactions	8- to 64-fold increase when antigen was injected into immunized rabbits or antigen-antibody complexes were injected into normal rabbits
Generalized Shwartzman reaction	8- to 32-fold increases in plasma β-lysin 6 h after endotoxin challenge
Bacteremia	4- to 16-fold increases in plasma β-lysin within 30 min after intravenous injection of bacteria

quence in vivo. It is also well known that a major problem in managing wounded patients is the control of infection at the wound site. The platelet β-lysin that would be released as a result of blood coagulation would be available in the wound site to participate in the control of such infection. The reactions in the blood coagulation sequence that are responsible for the release of platelet β-lysin have not been fully elucidated. Nevertheless, it is clear that platelet β-lysin is not released by the first two calcium-independent steps in the coagulation sequence because they proceed in the presence of chelating anticoagulants, which block release of platelet β-lysin (60). Likewise, the fibrinogen-to-fibrin reaction may be eliminated as a platelet β-lysin-releasing reaction because staphylococcal coagulase can drive this reaction in the presence of platelets and not release platelet β-lysin (60). In contrast, the prothrombin-to-thrombin step was a platelet β-lysin-releasing reaction; and thrombin alone was capable of releasing β-lysin from platelets, although its releasing activity was enhanced by other plasma factors (60). It is not known if the calcium-dependent steps in the coagulation sequence, which precede the formation of thrombin, are platelet β-lysin-releasing reactions. It is possible that platelet β-lysin is released with platelet factors 1 and 3, which are active in these reactions. Some support for this possibility is provided by the observation that a calcium-dependent platelet β-lysin-releasing reaction occurred in the presence of high concentrations of heparin, which blocked the prothrombin-to-thrombin conversion (38). However, the interpretation of this result was obscured by the fact that the platelet β-lysin-releasing reaction was stimulated by this anticoagulant (60). The higher the heparin concentration, the faster the platelet β-lysin release occurred.

Platelet β-lysin was either absent or present in concentrations too low to measure in washes of normal tissues of rabbits (33). However, high levels of platelet β-lysin accumulate rapidly in these same tissue sites as a consequence of the inflammatory process. Even a mild irritant like glycogen-saline stimulated a dramatic release of platelet β-lysin into the peritoneal cavity. During a 24-h period after administering glycogen saline, the level of platelet β-lysin in the peritoneal fluid increased from undetectable to 32 U/ml. The specific activity of platelet β-lysin increased steadily, and at the termination of the experiment the specific activity of the peritoneal fluid was 13.9 U of platelet β-lysin per mg of nitrogen or 126 times as high as plasma (33). This indicated that platelet β-lysin was being selectively accumulated in this site of inflammation. Such results could not be obtained if plasma proteins were simply diffusing into the cavity. Essentially the same results were obtained by injecting an irritant such as aluminum silicate into a subcutaneous site in a rabbit. In this case, a wash of the inflamed site yielded 8 U of platelet β-lysin per ml, with a specific activity of 13 U of platelet β-lysin per mg of nitrogen (33).

Trauma or tissue damage in humans can also result in dramatically increased levels of a serum bactericidin for *B. subtilis*. This substance is probably platelet β-lysin, although neutralization studies with specific anti-β-lysin have not yet been done. Myrvik and co-workers (50) reported that patients with myocardial infarctions, pneumonia, and certain types of cancer frequently had serum bactericidal levels against *B. subtilis* that were 8- to 16-fold higher than normal. These results emphasize the ability of the body to respond to tissue damage or to the threat of infection by releasing high levels of bactericidin.

The intravenous injection of bovine serum albumin into bovine serum albumin-immunized rabbits, or the injection of bovine serum albumin–anti-bovine serum albumin complexes into normal rabbits, increased plasma platelet β-lysin concentrations to near serum levels (59). Although this release appeared to be independent of the blood coagulation process, platelet disruption was taking place. The number of platelets in circulation decreased markedly, and the platelets remaining in the circulation appeared damaged when examined under the electron microscope (59). In vitro experiments suggested that this release may have been mediated by certain calcium-dependent reactions in the complement pathway. The addition of antigen to heparinized blood from an immunized animal, or the addition of antigen-antibody complex to heparinized blood from a normal animal, stimulated a rapid β-lysin release. Such additions to blood depleted of divalent ions by chelating agents would not stimulate the release of platelet β-lysin (54).

A single intravenous injection of 10 to 1,000 μg of endotoxin per kg into normal rabbits caused a marked decrease in the number of platelets in circulation, but did not alter the level of platelet β-lysin in the plasma. However, induction of a generalized Shwartzman reaction by a second endotoxin injection 24 h later stimulated a marked increase in plasma β-lysin. Kidney damage and fibrin clots are hallmarks of the generalized Shwartzman reaction, and this may be the mechanism responsible for releasing platelet β-lysin, although the release of platelet β-lysin did not correlate with the production of kidney lesions (61).

The injection of *Micrococcus lysodeikticus* into normal rabbits or the addition of these bacteria to whole blood containing an anticoagulant stimulated a release of platelet β-lysin. The mechanism of this release did not appear to depend upon the coagulation sequence or the complement pathway, since it occurred in the absence of calcium in citrated whole blood. Further experimentation indicated that the addition of micrococcal cells to platelet-rich plasma did not stimulate a platelet β-lysin release (59). However, the platelet β-lysin-releasing mechanism could be restored by adding peritoneal leukocytes, consisting of over 90% neutrophils, to the platelet-rich plasma (54). It was postulated, but not proven, that during phagocytosis a substance was released from the phagocytes that would stimulate the release of platelet β-lysin.

In view of the variety of pathways that will stimulate the release of platelet β-lysin, it was considered important to determine whether any enzyme that would act on the surface of a blood platelet might stimulate a β-lysin release. Eighteen different enzymes were studied to determine their platelet β-lysin-releasing abilities when incubated with platelet-rich plasma or injected in vivo (54). As predicted, enzymes like neuraminidase, papain, and phospholipase C released serum levels of β-lysin from platelets, whereas enzymes like elastase, phosphatase, and ribonuclease did not. However, other enzymes, like streptokinase and sulfatase, for which the platelet membrane is not an obvious substrate also released platelet β-lysin. The mechanism of release by these enzymes is not clear, but they may be activating other enzymes in the plasma which in turn act on the platelet. These data support the hypothesis that platelet β-lysin release is an enzyme-mediated process that occurs in vivo when intracellular enzymes are released or proenzymes are activated as a result of coagulation, phagocytosis, inflammation, or complement activation. The enzymes activated by each of these pathways could be very different as long as their specific substrate was available on the platelet surface. It is interesting that the streptokinase and phospholipase C are of bacterial origin. In an active infection these enzymes may directly release platelet β-lysin, and host enzyme systems may not be required.

In addition to active enzymes, platelet β-lysin may be released by a number of platelet-aggregating substances including collagen, arachidonic acid, and adenosine 5′-diphosphate (54). However, not all platelet-aggregating substances release platelet β-lysin. The addition of L-epinephrine, zymosan, fibrinogen, reserpine, and serotonin to platelet-rich plasma caused little or no release of platelet β-lysin (54).

SITE AND MODE OF ACTION OF PLATELET β-LYSIN

Studies of the viability and morphological and turbidity changes of both viable and nonviable bacteria and their components exposed to purified platelet β-lysin helped elucidate the site of action of platelet β-lysin. The overall effects of platelet β-lysin on different bacterial preparations are recorded in Table 6.

The cell wall was not considered to be the site of action of platelet β-lysin, even though cell wall damage for the first 24 h after platelet β-lysin treatment of *B. subtilis* was greater than that of untreated cells or cells killed by ultraviolet light. Cell walls were eliminated as the primary site of action of β-lysin by the following observations. (i) Within 2 min after exposure to β-lysin, most *B. subtilis* organisms were killed by platelet β-lysin, but lesions in the cell wall did not become visible with electron microscopy until about 2 h later (40). (ii) A slow drop in turbidity of platelet β-lysin-treated cell suspensions continued for hours after the rapid killing, which was almost complete within minutes (40). (iii) Neither the turbidity nor the electron microscopic appearance of a purified preparation of cell walls from *B. subtilis* was altered by platelet β-lysin treatment (41). (iv) Platelet β-lysin had no effect on the rate of breakdown of cell walls that takes place when cell wall preparations are not heated to destroy autolytic enzymes (41). These autolytic enzymes may be released from the cell

TABLE 6. *Effect of platelet β-lysin on bacteria and their components*

Test material	Action of platelet β-lysin
Viable *B. subtilis*	Immediate death accompanied the rupturing of cell membrane followed by lysis of the bacteria over the next 20 h
Viable *E. coli*	No death but extensive damage to the cell membrane resulting in an immediate loss of periplasmic space followed by recovery of bacteria
Protoplasts of *B. subtilis*	Immediate lysis
Protoplasts of *E. coli*	Immediate lysis
Ultraviolet-killed *B. subtilis*	Immediate rupture of cell membrane followed by slow lysis of bacteria over next 20 h
Purified cell walls of *B. subtilis*	None
Purified cell membranes of *B. megaterium*	Membrane aggregation accompanied by loss of unit structure of the membranes

and cause the slow breakdown of the cell wall that follows platelet β-lysin treatment.

The cell or plasma membrane is considered to be the primary site of action of β-lysin for the following reasons. (i) Platelet β-lysin prevented the development of the periplasmic space between the cell wall and the cell membrane, which normally develops when bacteria are suspended in hypertonic sucrose solutions (41). (ii) Protoplasts were readily ruptured by platelet β-lysin (41). (iii) Freeze-etched preparations of platelet β-lysin-treated *B. subtilis* have wrinkled and pitted membranes. With this technique, we have yet to see a normal cell membrane in a treated cell or a pitted membrane in an untreated cell. (iv) There was a loss of the unit structure of the membranes after platelet β-lysin treatment of purified membrane preparations (22). (v) Platelet β-lysin combined with and was absorbed out of solution by *B. megaterium* cell membrane preparations (22).

Escherichia coli, which were resistant to the lethal action of platelet β-lysin, appeared to have a cytoplasmic membrane that was damaged by this compound. As was the case with gram-positive bacilli, platelet β-lysin treatment of viable *E. coli* eliminated the periplasmic space (13). Furthermore, *E. coli* spheroplasts devoid of bacterial cell walls were ruptured by purified platelet β-lysin (13). Since *E. coli* were not killed by platelet β-lysin even though their cell membranes were extensively damaged, it appears that the cell walls of these gram-negative bacteria are a better protective barrier than the cell walls of gram-positive bacteria.

Factors indicating that β-lysin is not a bactericidal enzyme are: (i) the small molecular weight of β-lysin (34); (ii) β-lysin attaches to and is inactivated by the bacterial cell membranes (22); (iii) β-lysin is active over broad temperature and pH ranges. In unreported studies carried out in our laboratory, β-lysin was bactericidal in an ice bath or at 45°C. As the temperature or pH approached bactericidal levels, the β-lysin continued to be bactericidal until it could not be measured. Rosenthal and Buchanan (55) suggested that cationic bactericidal agents, which have no innate enzymatic activity, combine electrostatically with membrane anionic compounds such as phospholipids, causing changes that affect the membrane-bound enzymes. They demonstrated that platelet β-lysin, along with the other cationic bactericidal agents, histone, protamine sulfate, and cetyltrimethylammonium bromide, will stimulate the adenosine triphosphatase activity of the cell membranes of *B. subtilis*, whereas spermine, cadavarine, and putrescine inhibit enzyme activity. Buchanan and Yang (personal communication) found that β-lysin, histone, and cetyltrimethylammonium bromide also inhibited the membrane-bound reduced nicotinamide adenine dinucleotide oxidase and reduced nicotinamide adenine dinucleotide-dichlorophenolindophenol oxidoreductase. This could result in an interruption of electron flow and disruption of the respiratory chain. The significance of similar secondary phenomena and the activation or inactivation of membrane-bound enzymes in the killing by cationic agents is highly speculative at this time. If such an electrostatic attachment takes place between anionic bacterial membrane components and cationic polypeptides, the charge distribution of both the cidal agent and the bacterial membrane may be of primary significance for killing activity. Zeya and Spitznagel (65, 66) used a series of basic polypeptides with molecular weights of approximately 8,000 from polymorphonuclear leukocytes and showed that the bactericidal activity of a polypeptide was selective for the types of bacteria it would kill. Different polypeptides killed different types of bacteria. Such selectivity tends to rule out a general detergent effect as the primary means of killing by β-lysin and other cationic polypeptides.

COOPERATIVE INTERACTION AMONG PLATELET β-LYSIN, LYSOZYME, COMPLEMENT, AND ANTIBODY

The necessity for antibody and complement in killing gram-negative bacteria by serum has been known for over 80 years, but with few exceptions, the activity of the antibody-complement system was tested in the presence of other bactericidal agents found in normal serum. Treatment of serum with bentonite will remove both lysozyme (29) and platelet β-lysin (14). This treatment will also inhibit both the lysis (20, 29, 44, 62) and killing (21, 23) of gram-negative bacteria without depressing the serum complement or antibody concentrations (21, 29). The bactericidal activity of bentonite-treated serum was partially restored by the addition of lysozyme at high concentrations (15, 16, 21, 44). However, lysozyme even at 10 times the normal serum concentration did not completely restore the bactericidal activity of bentonite-treated serum (19). Glynn and Milne (21) postulated the existence of a bentonite-absorbable factor other than lysozyme that contributed to serum bactericidal activity. The fact that bentonite treatment removes platelet β-lysin as well as lysozyme suggested to us that the missing factor was platelet β-lysin (14).

Studies in our laboratory on the interrelationships among platelet β-lysin, lysozyme, and the antibody-complement system on the viability, turbidity, and morphological appearance of *E. coli* revealed or substantiated the following (13). (i) Inactivation of complement completely eliminated serum bactericidal activity for this organism even though the lysozyme and platelet β-lysin in the heated serum continued to cause morphological alteration in these organisms. (ii) The most efficient killing and destruction of these gram-negative organisms occurred when platelet β-lysin, lysozyme, and the antibody-complement system were functioning in cooperation with each other. (iii) The bactericidal activity, the turbidity reduction, and the morphological damage were identical to that of normal serum when both lysozyme and platelet β-lysin were added to bentonite-treated serum at physiological concentrations. (iv) The elimination of lysozyme from serum had the least effect, and the absence of platelet β-lysin had an intermediate effect on the destruction of *E. coli*. (v) The presence of these bactericidal systems alone and in conjunction with each other caused characteristic types of damage. As previously reported (15, 16), the antibody-complement system appeared to damage both the lipopolysaccharide layer of the cell wall and the cytoplasmic membrane (13). Lysozyme damaged the inner peptodoglycon layer of the cell wall, and platelet β-lysin damage appeared to be restricted to the cytoplasmic membrane. These results support the hypothesis that antibody, complement, platelet β-lysin, and lysozyme cooperate in mediating the destruction of gram-negative bacteria in vivo.

CONCLUDING COMMENTS

The exact role and quantitative significance of platelet β-lysin as a host defense mechanism remains to be established. It is clear, however, that platelet β-lysin is a remarkably potent bactericidal agent capable of killing millions of saprophytic bacteria, which continually come in contact with the human or animal body. The innate ability of living animals to resist invasion by such organisms is remarkable in view of the fact that animal tissue provides a complete source of nutrients and the fact that the physical and chemical conditions in the body such as temperature, pH, ionic strength, oxygen tension, and water content are nearly ideal for growth. Yet, in nature most bacteria rarely infect or invade living tissue. It is entirely possible that platelet β-lysin plays a major role in maintaining sterile conditions in the body by eliminating nonpathogenic bacteria such as *B. subtilis* before the infectious process can be established.

Recent work also makes it clear that platelet β-lysin is closely associated with such well-known defense mechanisms as inflammation, blood coagulation, phagocytosis, and antigen-antibody reactions. Platelet β-lysin may serve in these systems as a very important amplification mechanism. For example, it is clear that platelet β-lysin is capable of amplifying the antibody-complement-mediated killing and lysis of gram-negative bacteria. It is conceivable that in some cases the assistance afforded by platelet β-lysin may be necessary for the body to eliminate a given gram-negative organism. It should also be emphasized that in all of these host defense mechanisms, platelet β-lysin is released quickly and in high concentrations. It is entirely possible that these high levels of free platelet β-lysin are of major importance in the control of certain infections.

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