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Nonenteric Toxins of *Staphylococcus aureus*

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INTRODUCTION

Bacteria produce a multitude of extracellular products that display a wide range of biological activities. Many of these products are toxins. It is difficult to present a clear, precise definition of a bacterial toxin. This is because toxins vary so extensively in their physicochemical and biological properties. A striking example of the vast differences among bacterial toxins can be shown by way of a comparison between the lipopolysaccharide endotoxins of gram-negative bacteria and the extracellular protein toxins made by gram-positive bacteria. The principle common feature of bacterial toxins is that they are deleterious to specific hosts when administered in small doses.

Ideas about a relationship between pathogenicity and bacterial toxins are as old as the germ theory of disease. The ability of extracellular culture filtrates of *Staphylococcus aureus* to cause inflammatory reactions after inoculation into experimental animals was first reported in 1888 (112, 190). By 1900, it was known that diffusible substances of *S. aureus* affected erythrocytes and leukocytes. A major event that focused serious attention on the study of staphylococcal toxins was the Bundaberg disaster (23). In 1928, 12 out of 21 children who were inoculated with a diphtheria toxin-antitoxin preparation in Bundaberg, Australia, died. A bacteriological investigation suggested that a hemolytic toxin made by a strain of *S. aureus* within the unrefrigerated inoculum killed the children. This tragedy stimulated an increased awareness of the toxic and virulent properties of *S. aureus*. In subsequent years the existence of four staphylococcal hemolytic agents (alpha-, beta-, delta-, and gamma-toxin) was verified. These hemolytic agents were appropriately termed hemolysins. The hemolysins were differentiated on the basis of their lytic activity for different species of erythrocytes. As knowledge about the hemolysins grew, it became evident that these toxins were active on cells other than erythro-

cytes. Another staphylococcal extracellular toxin, leucocidin, affected leukocytes but not erythrocytes. To define the spectrum of the biological activity of the hemolysins more accurately, Bernheimer (17) introduced the term "cytolytic toxin." Cytolytic toxins (or cytolysins) were defined as bacterial products capable of causing physical dissolution of a variety of cells in vitro (17). However, low concentrations of cytolytic toxins will induce permeability changes in the membranes of intact susceptible cells without causing lysis (168, 169). Therefore, McCartney and Arbuthnott (112) have suggested that "membrane-damaging toxin" is a more descriptive term than cytolytic toxin to describe alpha-, beta-, delta-, and gamma-toxin. In any event, either cytolytic toxin or membrane-damaging toxin is a more appropriate term than hemolysin in describing the biological activity of alpha-, beta-, delta-, and gamma-toxin. Therefore, the term hemolysin, in reference to these toxins, should be retired with due recognition of its past distinction.

In 1878, Ritter von Rittershain (142) described a bullous exfoliative dermatitis in a large group of infants who were from a foundling home in Prague. In subsequent years, the impetigo was shown to be of staphylococcal etiology and became known as the staphylococcal scalded skin syndrome. In 1971, an extracellular staphylococcal product was isolated and was shown to produce the staphylococcal scalded skin syndrome after inoculation into neonatal mice (10, 84). The virulence factor was termed "exfoliatin" (84) or "exfoliative toxin" (121). This toxin is distinct from the membrane-damaging toxins in not inducing cell lysis and in being directly responsible for a specific disease.

The most significant studies pertaining to staphylococcal toxins have been concerned with production, purification, characterization, mode of action, genetic control, and relationship to pathogenicity. *S. aureus* can produce up to 30 different types of extracellular products (173). Many of these products are toxins and enzymes. Trace amounts of contamination by these products in specific toxin preparations, which were supposed to be pure, have been responsible for many misconceptions about a toxin's properties and behavior. Many misinterpretations about alpha-toxin resulted from data obtained with alpha-toxin preparations contaminated with delta-toxin (30, 42).

The most difficult problems that were encountered with studies on staphylococcal toxins were defining their mode of action and their role in pathogenicity. Alpha-toxin has been the most intensely studied staphylococcal toxin, yet its precise mode of action and role in pathogenicity are not completely understood. The extremely complex set of events that occur between leucocidin and the leukocyte membrane (201, 202) should give some insight about the difficulty in trying to define a toxin's mode of action. Although the role of exfoliative toxin in pathogenicity is quite clear, very little is known about the toxin's mode of action. To understand the mode of action of a staphylococcal toxin, it is essential to define the factors that govern the specificity of a toxin for receptor and target sites. This would explain why rabbit erythrocytes are approximately 100-fold more sensitive to alpha-toxin than human erythrocytes and why the skin of the neonatal mouse and of the human infant is so sensitive to effects of exfoliative toxin. The knowledge of how a toxin functions in vivo can provide a molecular interpretation of how toxins contribute to pathogenesis. Yet, with the exception of beta-toxin, we do not fully comprehend the basis for the specificity of staphylococcal toxins for biological sites. Beta-toxin is a sphingomyelinase. Therefore, a cell's susceptibility to beta-toxin is directly related to the amount of membrane sphingomyelin which becomes accessible to the toxin. Among all the other staphylococcal toxins, only beta-toxin appears to have an enzymatic mode of action. Therefore, interaction of the other staphylococcal toxins with host receptors probably involves mechanisms that are more difficult to recognize than an obvious enzyme-substrate reaction. However, the action of alpha-toxin may involve enzymatic activity (190).

During the past few years there has been an increasing interest in studying the genetic control of staphylococcal toxins. *S. aureus* produces serologically distinct types of enterotoxins and exfoliative toxins. It is not unusual for a staphylococcal strain to produce more than one type of the same toxin. In such a circumstance, one toxin might be controlled by a chromosomal genetic determinant and the other by an extrachromosomal genetic determinant (146). In addition, it would not be highly speculative to suggest that staphylococcal toxins might be associated with transposons.

This review will only devote a limited amount of discussion to the production, purification, and pharmacological effects of the membrane-damaging toxins. A major objective of this manuscript will be to review the current status of knowledge about the molecular mechanisms which involve the interaction of alpha-, beta-, delta-, and gamma-toxin and leucocidin with biological membranes. Special attention will be given to a discussion of the current status of knowledge about exfoliative toxin. Within the past few years a number of investigators have

independently isolated and purified serologically distinct types of exfoliative toxins. The properties of these toxins have been defined, and progress has been made toward understanding their mechanism of action. Other recent studies (146, 185) have defined the genetic regulation of exfoliative toxin synthesis. Therefore, there is a need to bring together the scattered data on exfoliative toxin research into an orderly and organized review. A review of the research with exfoliative toxin coupled with the recent advances made with other nonenteric staphylococcal toxins will serve an important function in broadening our comprehension of bacterial pathogenesis. *S. aureus* serves as an ideal model system for studying the interaction between bacterial toxins and pathogenicity. This is because staphylococci produce a large number of different toxins and cause disease in nearly every organ of the human body.

STAPHYLOCOCCAL SCALDED SKIN SYNDROME

In 1878, Ritter von Rittershain (142) reported 297 cases of a skin affliction manifested by a bullous exfoliative dermatitis in infants less than 1 month old, whom he had observed over a 10-year period. The dermatitis, now known as Ritter's disease, starts with an abrupt onset of a general localized erythema that begins periorally (Fig. 1) and spreads to cover the entire body within 2 days. These signs are usually preceded by a purulent conjunctivitis or upper respiratory infection (45). The early stages usually include exudative lesions with yellow crusty deposits. Although the skin appears intact, the epidermis becomes irreversibly wrinkled after gentle stroking and can be displaced with slight pressure (positive Nikolsky sign). Shortly thereafter, large flaccid bullae filled with clear fluid appear. These vesicles of sterile, clear fluid then lift large areas of the epidermis that separate and peel revealing the moist red glistening corium beneath (120) (Fig. 2). One-half or more of the body may become denuded. Ritter von Rittershain's description of the disease at this stage included the statement that the skin appeared scalded. The exfoliated areas dry and develop into large seborrhea-like flakes (119, 120). The flaky desquamation continues for 3 to 5 days during which there is rapid replacement of epithelium. Seven to 10 days after the first signs of erythema, recovery is complete with a normal unscarred appearance of the skin. Although Ritter von Rittershain never considered the disease he described to be infectious, Winternitz (187) isolated *S. aureus* from a patient with Ritter's disease in 1898. In succeeding years numerous

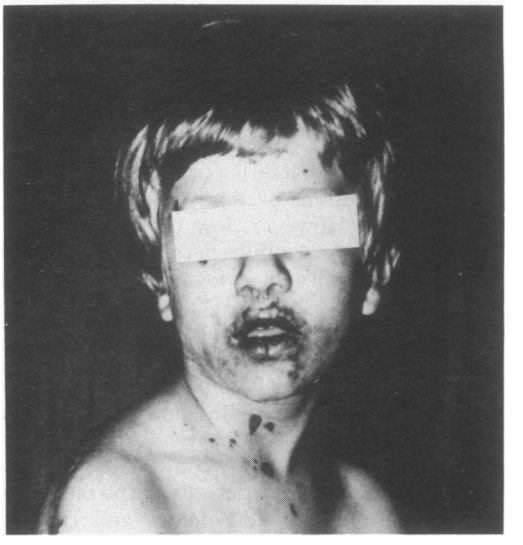

FIG. 1. *A 4-year-old patient with the scalded skin syndrome. Note the severe perioral erythema accompanied by exudative lesions. The impetigo was associated with phage group 2 staphylococci which were isolated from both a purulent conjuctivitis and a nasopharyngeal focus of infection in this patient. (Photograph provided by David Cram.)*

investigators provided overwhelming evidence which showed that Ritter's disease was staphylococcal in origin.

In 1956, Lyell (106) and Lang and Walker (99) independently described a toxic epidermal necrolysis (TEN) in adults that had a striking similarity to Ritter's disease. However, TEN, or Lyell's disease, was soon differentiated into two distinct subtypes. One type was nearly always confined to adults or older children and appeared to be caused by hypersensitivity to drugs such as pyrazolone derivatives, antibiotics, barbiturates, and sulfa compounds (157, 163). The second type of TEN is seen in infants and young children and is associated with staphylococci.

Histologically, staphylococcus-induced TEN is characterized by intraepidermal splitting through the stratum granulosum which is identical to that seen in Ritter's disease. Drug-induced TEN is characterized by splitting at the dermoepidermal junction (106, 211). Koblenzer (89) provided evidence which showed that staphylococcus-induced TEN was Ritter's disease rediscovered. Therefore, it has been proposed that staphylococcal exfoliative disease of the newborn be referred to as Ritter's disease and that this same syndrome, when occurring in children or adults, be referred to as TEN. TEN may or may not have a staphylococcal etiology; but when it does, it is referred to as Ritter's type

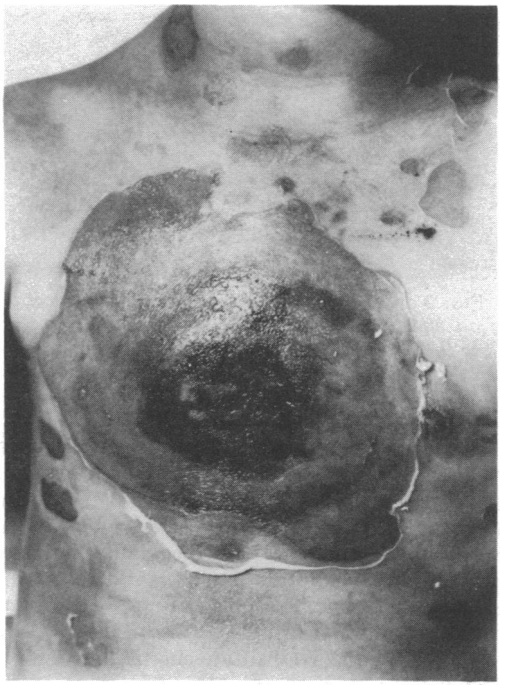

FIG. 2. *Generalized scalded skin syndrome in an 8-year-old female. Note the extensive exfoliation and peeling of the epidermis in an axial distribution that is characteristic of this disease. Although there was prompt improvement of this patient after antibiotic chemotherapy, the primary focus of infection was never found. (Photograph provided by Peter M. Elias.)*

of TEN. A small number of cases of Ritter's type of TEN have been reported in adults (43, 47, 49, 67, 100, 139, 182). However, nearly all of these cases involved some predisposing factor such as alcoholism, immunosuppression, or compromised cell-mediated immunity that may have played a role in the development of the disease.

Aside from TEN and Ritter's disease, other dermatological diseases caused by *S. aureus* include a bullous impetigo and a scarlatiniform rash. Staphylococcal bullous impetigo (47, 120) is characterized by flaccid localized bullous lesions on normal-appearing skin. A positive Nikolsky sign is not present. Most lesions spontaneously rupture, revealing a moist red surface beneath, but exfoliation does not reach beyond the margin of the original bulla. Staphylococci can be cultured from ruptured bullae. Bullous impetigo appears to be a clinically distinct entity among the impetigous diseases associated with *S. aureus* (120). The staphylococcal scarlatiniform rash is indistinguishable from the one in streptococcal scarlet fever. It produces a generalized erythema similar to that described for Ritter's disease, but without bullae or exfoliation. After 1 to 2 days of erythema, cracks appear in the skin creases that soon become covered by thick flakes of dried skin that spread to involve nearly the entire epidermis (120). After 4 to 5 days, these flakes desquamate revealing the healed skin beneath. Presently, it is believed that this type of staphylococcal dermatitis is a modified form of TEN (163). It is now important to emphasize that the spectrum of staphylococcal impetigous diseases which include Ritter's disease, TEN, bullous impetigo, and scarlatiniform rash comprise a syndrome referred to as the staphylococcal scalded skin syndrome (SSSS) (119, 120). Clinically, the skin lesions produced during the different variations of the single syndrome form an overlapping spectrum of disease. The localized lesions of bullous impetigo, Ritter's type of TEN, and Ritter's disease are clinically and histologically identical. Staphylococcus-induced TEN, Ritter's disease, and staphylococcal scarlatiniform rash are all generalized and have identical initial phases of erythema and skin tenderness and indistinguishable terminal phases of desquamation and healing.

Melish and Glasgow (119, 120) identified phage group 2 *S. aureus* as the sole etiological agent for SSSS. However, it is now known that the syndrome can be caused by strains of *S. aureus* belonging to phage groups other than group 2. In 1955, Parker et al. (135) were the first to demonstrate an association between phage group 2 staphylococci and SSSS. They also reported that 75% of these phage group 2 strains belonged to phage type 71. It has since been documented by numerous investigators that phage group 2 strains, especially of the type 71 variety, were isolated from patients with SSSS (1, 16, 33, 70, 84, 105, 119). Strains of phage groups other than group 2 are very rare in causing SSSS in the United States; however, such cases are occasionally reported (115, 138). In 1975, Rasmussen (138) found that 1 out of 53 phage-typed strains isolated from children with SSSS was of the phage group 1 variety. Also, in 1975 our laboratory received four phage group 1 strains isolated from neonates with SSSS during a nursery outbreak at Primary Children's Hospital in Salt Lake City, Utah. After working with these strains, we observed that they produced very weak or mild exfoliative reactions in newborn mice in comparison to our phage group 2 strains. In England, Abuthnott and Billcliffe (4) reported that, out of 11 SSSS-producing strains isolated from hospital infections, 3 did not belong to phage group 2. In Japan, it appears that clinical staphylococcal isolates from patients with SSSS commonly show a typing pattern

other than phage group 2. Kondo et al. (93) showed that, out of 43 SSSS-producing strains isolated in Japan, 24 were phage group 2 strains and 19 were typed as either phage group 3 or as a combined phage group 1 and 3 variety. Another Japanese survey revealed that approximately 80% of a patient population with Ritter's type of TEN carried non-phage group 2 staphylococci (151). It is of particular interest that, up until a few years ago, only group 2 organisms were isolated from patients with SSSS in Japan (151). Further comparative surveys on the distribution of *S. aureus* phage types that produce SSSS in different countries might yield interesting results.

Experimental Model

In 1970, Melish and Glasgow (119) discovered that clinical isolates of staphylococci could reproduce SSSS in neonatal mice. This significant discovery firmly established the etiology of the disease syndrome. The availability of the neonatal mouse model presented an ideal tool to aid in determining how staphylococci cause SSSS. Seventeen phage group 2 staphylococcal strains isolated from patients with various forms of SSSS were capable of producing extensive exfoliation after inoculation into mice less than 6 days old (Fig. 3). The organisms were grown for 24 h in Trypticase soy broth, and 0.1 ml of serial 10-fold dilutions was inoculated with a 25-gauge needle either by the subcutaneous or by the intraperitoneal route. Elicitation of SSSS in mice was dose dependent. For each strain capable of producing an exfoliative reaction, a specific titer of bacteria was found to be lethal to all neonatal mice within 10 h after injection. The animals died without any noticeable dermatitis, and their death was most likely caused by a staphylococcal alpha-toxin. The lethal dose ranged from 10^7 to 10^{10} organisms. An approximate 10-fold dilution of the lethal dose (effective dose) produced a reaction in neonatal mice that remarkably simulates the sequence of clinical events which occurs in the fully developed human syndrome. Further 10-fold dilutions gave subeffective doses that failed to produce signs of the syndrome in mice. Staphylococci inoculated by the intraperitoneal route produced a typical exfoliative reaction, but the effective intraperitoneal dose was usually 10 times higher than the effective subcutaneous dose. Twelve to 16 h after injection, a positive Nikolsky sign developed, first on the back and then on the abdomen. Four hours later bullae and spontaneous epidermal wrinkling appeared, followed by extensive exfoliation. Denuded skin surfaces were positive for staphylococci, but aspirated fluid from intact bullae was always sterile (119).

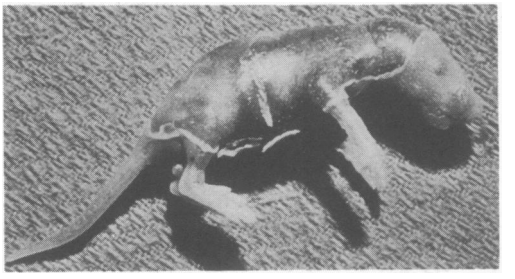

FIG. 3. *Response of a neonatal mouse to injection of an effective dose of ET-producing staphylococci. Note the extensive epidermal exfoliation that covers nearly the entire body surface.*

Twenty hours after subcutaneous inoculation of SSSS-producing staphylococci, clusters of cocci were seen deep in the dermis beneath the superficial muscle layer at the site of inoculation. Surprisingly, there was no local inflammatory response (119). Kapral (82) observed that staphylococci capable of eliciting SSSS were different from conventional strains because of their ability to multiply in the subcutaneous tissue of mice. The histological pattern in the experimental syndrome was identical to that reported for the human syndrome.

Association of the Staphylococcal Scalded Skin Syndrome with Exfoliative Toxin

The ability to induce exfoliation after intraperitoneal inoculation of staphylococci as well as the inability to isolate organisms from intact bullae suggested that SSSS might be caused by an extracellular staphylococcal product. This hypothesis was supported further by the histological evidence which shows that the etiological organisms are concentrated and multiply at a site distant from the typical lesions of the disease syndrome (82). In addition to Melish and Glasgow (119), Lowney et al. (105) and Jefferson (72) also postulated that the manifestations of SSSS were due to a soluble product of staphylococci. It was first thought that this soluble product might be delta-toxin. Arbuthnott et al. (9) speculated that, if delta-toxin were produced in vivo, the toxin might aid in establishing or perpetuating lesions associated with SSSS. As a result of the ongoing studies with delta-toxin and the availability of the experimental model, investigators from two separate laboratories (10, 84) attempted to identify the product responsible for SSSS and, independently, made discoveries that equaled the importance of the development of the animal model. Arbuthnott and co-workers (10) incubated an alpha-toxin-negative phage type 71 organism that was isolated from a 10-

year-old child with TEN for 48 h in semisolid nutrient agar in an atmosphere of 20% CO_2. The extracellular fluid from the culture was dialyzed against 0.05 M sodium acetate at a pH of 4.0. After adsorption chromatography on hydroxyapatite, a thermolabile product capable of causing generalized exfoliation in newborn mice was partially purified. The product, or "epidermolytic toxin," caused the same clinical and histological changes in the epidermis as those described for viable staphylococci after injection into newborn mice (Fig. 4). Other workers (46, 182) later showed that the toxin behaved similarly after injection into human skin, which indicated that the toxin alone was capable of eliciting SSSS.

Kapral and Miller (84) discovered independently that certain phage group 2 *S. aureus* strains produced a protein distinct from alpha- and delta-toxins which was capable of producing exfoliation of neonatal mice and which was responsible for SSSS in humans. This protein was named "exfoliatin." Kapral (80) later observed that, out of 200 phage group 2 strains examined, 40% were capable of producing exfoliatin in vitro. In another survey (80) involving 1,000 randomly selected *S. aureus* strains, 19 non-group 2 strains were found to produce exfoliatin, which showed that toxin production was not limited to phage group 2 strains. Other laboratories have also documented that the toxin is made by non-group 2 strains (4, 93, 151, 185). The same presumed extracellular product responsible for SSSS has been referred to as either epidermolytic toxin or exfoliatin. Other workers referred to this toxin as epidermolysin (36) or exfoliative toxin (121). In this review the toxin will be designated exfoliative toxin, or ET. In our laboratory (unpublished results), it has been observed that some strains producing ET in vitro did not elicit a positive Nikolsky sign after inoculation into newborn mice. This was most often observed with non-group 2 strains. Since most patients with SSSS lack primary localized lesions that contain *S. aureus*, it is assumed that the impetigo is caused by dissemination of toxin coming from organisms carried in primary foci of the upper respiratory tract, eyes, or ears (80). Differences in the amount or type of toxin produced by the organism and in the mode of toxin adsorption by the host may be factors that determine whether a susceptible host responds with either the scarlatiniform rash, bullous impetigo, extensive exfoliation, or a negative reaction.

EXFOLIATIVE TOXIN

Production

Many laboratories routinely prepare ET.

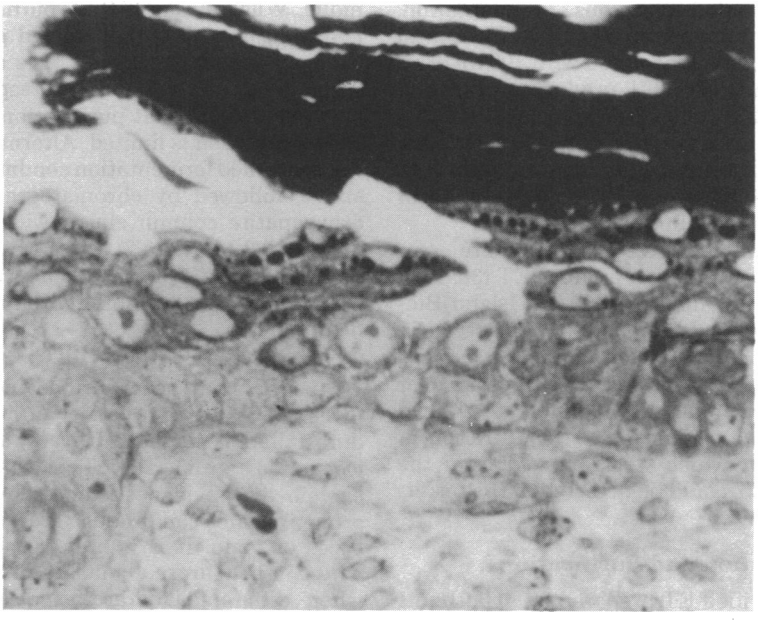

FIG. 4. *Photomicrograph showing the histopathological characteristics of the skin of a neonatal mouse 2 h after injection with ET. Note the typical cleft formation that occurs after toxin-induced cleavage through the stratum granulosum. The cleavage plane appears to be intercellular and there is no evidence of lysis or cytotoxicity of granular cells along the cleavage plane. Magnification, ×1,600. (Photomicrograph provided by Peter M. Elias.)*

They utilize a variety of methods for the production of ET which range from in vivo production in the peritoneal cavity of rats to in vitro production under different cultural conditions. It has already been mentioned that one method of toxin production involves incubation for 48 h in semisolid nutrient agar under 20% CO_2 (10). However, this did not prove to be a popular method as most other laboratories preferred the use of liquid culture media. Kapral and Miller (84) prepared ET in a medium containing 10 g of yeast extract, 17 g of Trypticase, 5 g of NaCl, and 2.5 g of K_2HPO_4 per liter of water. Cells were grown with continuous agitation for 48 h in a 10% CO_2 atmosphere. This method of ET production was adopted by other laboratories (77, 90). Wiley et al. (184), using four different strains which produce toxin, attempted to produce ET in a number of different broth media under approximately 10 to 20% CO_2 and found that cells incubated with continuous agitation in heart infusion broth produced optimal quantities of ET. Although minute quantities of toxin could be detected in the supernatant fluid during the log phase of growth, maximum titers were not attained until 48 to 96 h after incubation. The cultural conditions described above were limited to the production of only milligram amounts of ET. However, Johnson et al. (77) were successful in obtaining gram amounts of ET under controlled conditions of fermentation. Fifty liters of the medium described by Kapral and Miller (84) was inoculated with 100 ml of an overnight culture of an ET-producing strain and incubated for 20 h in an atmosphere of 10% CO_2 in a 70-liter fermentor agitating at 400 rpm. After concentrating the culture supernatant fluid with an Amicon TC3E system, a yield of 14.5 g of crude toxin was obtained.

Other laboratories have used the more cumbersome in vivo method to produce ET (36, 121). Tissue culture medium 199 (Grand Island Biological Co., Grand Island, N.Y.) was placed in sterile dialysis sacs, inoculated, and introduced surgically into the peritoneal cavity of a rat or rabbit. After 2 to 5 days the animals were killed and the sacs were removed. After filtration of the milky fluid which was removed from the dialysis sacs, ET could be detected in the resulting clear fluid.

Isolation and Purification

Partially purified ET prepared by Arbuthnott et al. (10) was found to contain delta-toxin. The most difficult problem encountered in the purification of ET was freeing it from alpha-toxin. Arbuthnott et al. (10) circumvented this problem by using an alpha-toxin-negative strain. Kapral and Miller (84) used diethylaminoethyl-Sephadex chromatography with 0.1 M ammonium acetate buffer (pH 9.0) to isolate pure alpha-toxin-free preparations of ET. Kondo et al. (90) achieved similar results by passing an ammonium sulfate-precipitated preparation of ET dissolved in a 0.01 M tris(hydroxymethyl)-aminomethane hydrochloride buffer (pH 7.5) through a Sephadex G-75 column equilibrated and eluted with the same buffer. The preparation was then passed through diethylaminoethyl-cellulose, equilibrated with the same tris(hydroxymethyl)aminomethane hydrochloride buffer, and eluted with a linear gradient from 0 to 0.2 M NaCl. Dimond and Weupper (36) were able to separate ET from alpha- and delta-toxin by zone electrophoresis in Pevikon at a pH of 9.0, followed by cation exchange chromatography on carboxymethyl-Sephadex G-50 to remove trace contaminants. In other studies (5, 121, 184), isoelectric glycerol density gradient electrophoresis proved to be an adequate method for purification of ET. A major advantage of this technique is that ET, with an isoelectric point of approximately 6.8, and alpha-toxin, with an isoelectric point of approximately 8.5, are easily separated. A disadvantage of this method is the tedious effort of removing the glycerol and carrier ampholytes from the pools of focused ET. Utilizing the electrofocusing technique, Wiley et al. (184), Arbuthnott et al. (5), and Melish et al. (121) detected purified ET in fractions focusing between pH's of 6.0 and 6.9, 6.0 and 7.2, and 6.5 and 7.3, respectively. The amount of toxin that purification by electrofocusing can yield is limited. Alternately, by using the controlled fermentation conditions described above, followed by chromatography on a hydroxyapatite column eluted with a linear gradient of 0.2 M (pH 5.7) to 0.4 M (pH 5.7) phosphate, Johnson et al. (77) applied a scheme that produced virtually unlimited amounts of purified toxin.

Properties

Since ET has been purified by a number of investigators using a variety of isolation procedures, there is much controversy about its physicochemical properties. This can be partially explained by evidence which shows that phage group 2 strains can produce at least two distinct types of toxin. It is, therefore, understandable why conflicts arose about the properties of ET when different laboratories, assuming themselves to be analyzing the same product, were actually working with either different toxins or a mixture of two or more products with exfoliative activity.

ET is antigenic and believed to be protein in

nature. Its biological activity is lost after interaction with either trypsin or Pronase (84). Amino acid analyses of a purified ET preparation showed that the protein was rich in aspartate, glutamic acid, and glycine and consisted of 17 amino acids (77). Arbuthnott et al. (11), using a phage group 2 strain, purified ET that had an isoelectric point of 6.7, a molecular weight of 33,000, and was thermolabile. ET isolated from a group 2 strain by Kapral and Miller (84) was a thermoresistant protein with a molecular weight of 23,500 and an isoelectric point of 4.0. A phage group 2 toxin isolated by Kondo et al. (90) had similar properties to the protein isolated by Kapral and Miller (84). In addition, Kondo et al. (90) reported that the ET from the phage group 2 strain ZM could be separated into four bands after discontinuous electrophoresis on 7.5% polyacrylamide gels. Although the four ET components had different levels of biological activity and electrophoretic mobilities, they were identical with respect to antigenicity and other physicochemical properties. Since later studies by Kondo et al. (93) showed that strain ZM synthesized only one distinct type of toxin, it is assumed that a single species of ET can have multiple polymeric forms with different electrophoretic migration patterns. Purified preparations of ET, isolated by Melish et al. (121) from a phage group 2 strain, were reported to be thermoresistant and to have a molecular weight of 33,000 and an isoelectric point of 7.0. Dimond and Weupper (36) isolated a phage group 2 ET that had a molecular weight of 28,600, as determined by sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis, and 32,500, as determined by sedimentation equilibrium on the analytical ultracentrifuge. The purified preparation, after electrophoresis on polyacrylamide gels, dissociated into two molecular forms that were identical in molecular weight and antigenicity but differed in electrophoretic mobility. The properties of some ET preparations, as reported by different investigators, are summarized in Table 1.

The data in Table 1 show differing reports for the molecular weight, isoelectric point, and heat lability of ET. The use of different group 2 strains, isolation procedures, and methods for molecular weight determinations could account for the observed differences in molecular weight. It has already been mentioned that Dimond and Weupper (36) obtained two different molecular weights for the same ET preparation by using two different methods for calculation. Certain isolation procedures could cause the toxin to complex with other components that would make the toxin appear to have a higher molecular weight. For example, purified preparations of ET, isolated in our laboratory by isoelectric density gradient electrophoresis, were glycoproteins containing 9% carbohydrate (147). B. Wiley (unpublished results) showed that the carbohydrate was not a contaminant from the glycerol density gradient used for electrofocusing. The carbohydrate could be disassociated from the glycoprotein by precipitation with concanavalin A without any loss of toxin activity in the solution. The suggestion that toxin isolated by isoelectric focusing is complexed with a carbohy-

TABLE 1. *Physicochemical properties of some phage group 2 staphylococcal ET preparations*

Strain for toxin source	Main purification step[a]	Mol wt	pI	Thermal tolerance[b]	Reference
G20	Isoelectric focusing	33,000	6.7	S, 60°C for 30 min	Arbuthnott et al. (11)
TG	DEAE-Sephadex chromatography	23,500	4.0	R, 60°C for 20 min	Kapral and Miller (84)
ZM	DEAE-Sephadex chromatography	24,000	4.0	R, 60°C for 60 min, or R, 100°C for 20 min	Kondo et al. (90)
UT0002	Isoelectric focusing	32,500	6.8	R, 60°C for 30 min	Wiley et al. (184)
TA	Isoelectric focusing	30,000	7.0	R, 56°C for 20 min	Melish et al. (121)
TA	Hydroxyapatite and CM-Sephadex chromatography	26,000	—[c]	—	Johnson et al. (77)
EV	CM-Sephadex chromatography	28,600[d] 32,500[e]	—	—	Dimond and Wuepper (36)

[a] DEAE, Diethylaminoethyl; CM, carboxymethyl.
[b] Symbols: S, sensitive; R, resistant.
[c] —, Not reported.
[d] Determined after analysis by SDS-acrylamide gel electrophoresis.
[e] Determined after sedimentation equilibrium analysis on the analytical ultracentrifuge.

drate might also explain why ET preparations isolated by this method are higher in molecular weight than preparations isolated by other methods (Table 1).

The observed differences in isoelectric points for ET could be explained by toxin separation into different conformational forms during electrophoresis. Several laboratories (5, 11, 121, 184) have detected multiple molecular forms from a single species of ET after isoelectric focusing, but the main component appeared to have an approximate isoelectric point of 6.8. It is common for extracellular proteins of *S. aureus* to exist in multiple conformational forms. These forms can be readily separated by electrophoresis on density gradients (173). Finally, the observed difference in the thermolability of ET (Table 1) can best be explained by the production of two antigenically distinct toxins by phage group 2 strains. Two serologically distinct forms of ET made by the same phage group 2 strain were first isolated by Kondo et al. (93) and referred to as exfoliatin A and exfoliatin B. A similar observation was made in two other laboratories. Wiley and Rogolsky (185) identified two serotypes of ET in phage group 2 strain UT0002 after double-diffusion precipitation analyses with electrofocused toxin and referred to them as ET II A and ET II B. Arbuthnott and Billcliffe (4) concluded, on the basis of radial immunodiffusion and double-diffusion precipitation tests, that two serologically distinct antigenic components (ET_A and ET_B) were present in four different phage group 2 strains. Although never proven experimentally, comparative data indicated that exfoliatin A, ET II A, and ET_A are different names for the same toxin and that exfoliatin B, ET II B, and ET_B are different names for the second type of toxin. It will be convenient, therefore, to refer to the former toxin as ET A and the latter as ET B. Kondo et al. (91, 92) have shown that, although ET A and ET B have the same molecular weight, the former was heat stable and latter was heat labile. ET A was stable upon storage at −30°C for 1 year, and ET B was inactivated within 20 days at this temperature (92). In addition, ET A but not ET B loses biological activity after treatment with 200 mM ethylenediaminetetraacetic acid at 4°C for 2 to 3 days (92). Ethylenediaminetetraacetic acid-treated ET A was reactivated after the addition of Mg^{2+}, Co^{2+}, Zn^{2+}, or Ni^{2+}. Radioactivation analyses by Sakurai and Kondo (149) confirmed that ET A was a metallotoxin. It was shown that one molecule of ET A contained one molecule of copper that was essential for toxin activity. Magnesium, cobalt, and zinc could be substituted for copper without affecting toxicity. The properties of ET A and ET B are summarized in Table 2.

In surveys screening for the distribution of the two toxin types among group 2 toxin-producing staphylococci, an individual strain was found to have either one or both of the toxin types. In one survey, Kondo et al. (92) found that ET isolated from strains that type as group 1, group 3, or a combination of groups 1 and 3 is nearly always of the ET B variety. Out of 17 non-phage group 2 strains tested in this survey, 2 produced ET A and ET B and 15 produced only ET B. ET A was clearly distinguished from ET B in these studies by double-diffusion precipitation tests. Another survey by Sarai et al. (151) revealed that there was no relationship between a phage typing pattern and the type of toxin produced. Experiments by Wiley and Rogolsky (185) revealed that two non-group 2 strains synthesized only the type B antigenic variety of toxin, but, in addition, these experiments also revealed some interesting new findings. Both a

TABLE 2. *Comparative properties of ET A and ET B*

Property	ET A	ET B	Reference
Mol wt	24,000	24,000	Kondo et al. (91)
Thermal tolerance	Stable after heating at 100°C for 20 min	Inactivated after heating at 60°C for 30 min	Kondo et al. (91)
Inactivated by EDTA[a]	Positive	Negative	Kondo et al. (92)
Cu^{2+} essential for biological activity	Positive	Negative	Sakurai and Kondo (149)
No. of electrophoretic variants observed after discontinuous gel electrophoresis	4	1	Kondo et al. (91)
Stability at −30°C	Stable after 1 yr	Inactivated within 20 days	Kondo et al. (92)
Location of the gene(s) for toxin synthesis[b]	Chromosomal	Extrachromosomal	Rogolsky et al. (146); Wiley and Rogolsky (185).

[a] EDTA, Ethylenediaminetetraacetic acid.
[b] Refers to phage group 2 strains only.

phage group 1 and a phage group 3 strain each produced one type of ET which had a reaction of identity with antiserum made against phage group 2 ET B. However, the toxin from the phage group 3 strain differed from that made by the phage group 2 strain in being thermoresistant and having a slightly lower molecular weight. The toxin produced by the phage group 1 strain was difficult to analyze since the strain produced very low quantities of toxin. This might account for the extreme difficulty in producing the disease syndrome after inoculation of the group 1 strain into neonatal mice. In summary, strains which make toxin synthesize at least two antigenically distinct types of ET which differ in physicochemical properties. There appears to be no clear relationship between a specific phage group and the type of ET made.

Ouchterlony double-diffusion precipitation tests (92) clearly indicated that at least two distinct species of antibody are made against the ET produced by staphylococcal phage group 1, 2, and 3 strains. In general, antibodies were produced in rabbits which received purified ET. Kapral (80) has reported that alum-precipitated ET is a much better immunogen than soluble ET. Johnson et al. (77) and Wiley and Rogolsky (185) initially mixed a 0.5-ml volume of solution containing 1 mg of toxin with 0.5 ml of Freund complete adjuvant and inoculated the mixture subcutaneously into New Zealand white rabbits. Subsequent injections containing 1 mg of toxin were then given at weekly intervals without the adjuvant. Test bleedings to recover antibody were done 7 days after the third inoculation. Most of the early work dealing with antibody production is difficult to interpret since some workers were not aware of whether they were producing antiserum against either ET A, ET B, or a combination of the two toxins. Wiley and Rogolsky (185) observed a weak response of rabbits to injection of purified ET B. Injections had to be continued for 4 to 5 months to yield antisera that gave a precipitin band in Ouchterlony double-diffusion tests. In some cases, up to 15 mg of purified ET was required to yield moderately reactive antisera.

Antibodies to ET appear to be protective and neutralizing in mice and humans (46, 116, 123, 183, 211) Rabbit antibody to ET either passively administered to mice (116, 123) or mixed with ET prior to intracutaneous injections (46, 183) into human volunteers consistently abrogated the biological action of ET. Although these experiments were performed without regard to whether anti-ET A or anti-ET B antisera were involved in protection, it seems likely that the neutralizing antibody to both serotypes of ET may play a significant role in preventing SSSS in human adults. Small doses of purified ET (0.3 to 6 μg), to which normal adult humans show no response, were shown to produce bullae in an adult human who lacked antibody to this ET preparation (80). Out of 64 randomly selected human sera from individuals of varying ages, 73% had antibody against an ET preparation from strain UT0002 that produces both ET A and ET B (183). In another study it was observed that antibody to ET is rarely observed in children with SSSS at the time of admission, but approximately 70% had increased antitoxin levels during the convalescent period (14). This observation led Baker and co-workers (15) to conclude that SSSS occurs in immunologically lazy or immunosuppressed individuals unable to respond promptly to ET by production of neutralizing antibody. The antitoxin was believed to play an important role in the prevention and/or termination of SSSS. Although it is presently evident that a normal immune state protects human adults from SSSS, the extreme rarity of the disease in individuals over the age of 5 years led Elias et al. (45) to believe that other factors might be involved. Biological and biochemical changes in adult epidermis might provide protection against ET. Since ET, even in large doses that are systemically administered, does not produce exfoliation in normal adult mice (48), Elias et al. (45) suggested by this indirect evidence that the adult human is better able to metabolize the toxin than are children. Furthermore, experiments designed to detect the distribution of iodinated ET in the blood and urine of mice indicated that the toxin is excreted rapidly by adult, but not neonatal, mice (56). If these observations are valid, it seems likely that future cases of SSSS in adults should be reported in metabolically deficient patients as well as in immunologically compromised individuals.

Sarai et al. (152) collected the sera from 200 healthy children (ages 1 to 5 years) and 200 healthy adults and tested them by the passive hemagglutination test for the presence of antibodies to ET A and ET B. The distribution patterns for the presence of ET A and ET B antibody were remarkably similar in both groups of human volunteers. Positive tests for ET A antibody were detected in 20% of the children and 17.5% of the adults. Positive tests for ET B antibody were detected in 4% of the children and 2.5% of the adults. As a result of the similarity in antibody titers for adults and children, Sarai et al. (152) suggested that susceptibility to SSSS in children might depend on factors other than lack of humoral antibody to ET.

Although little is known concerning the precise mechanism of action of ET, much interesting and plausible speculation on this topic has been presented. Nearly all the resultant theories have been based on observations of histological changes in susceptible epidermal tissue treated with ET. Any speculation as to the nature of the mechanism of action of ET must take into account the greater susceptibility of neonatal mice and infants as compared to adults.

Epidermal tissue in newborn mice is organized into the same layers as human skin. The epidermal cells are cemented to one another by a viscous matrix believed to consist of polymerized mucopolysaccharide. In 3-day-old mice the stratum granulosum appears to contain no less than three cell layers (116). Granular cells of newborn mice contain keratohyalin granules, Odland bodies, and structures at the apical poles which were referred to by McLay et al. (116) as compound granules. Compound granules differed from typical Odland bodies (membrane coating granules in peripheral cytoplasm) by being vesicular rather than lamellated. Compound granules were observed in the stratum granulosum of mice as old as 18 days. However, these mice contained more typical Odland bodies compared to younger mice which suggests that the compound granule of the neonatal mice might perform a function similar to that proposed for the Odland bodies. Individual granular cells are attached to one another by specialized thick cell membrane structures called desmosomes (101). There are small vesicles between cells of the stratum granulosum that reveal light-density material which show oval profiles or "bubbles" (101). Lillibridge et al. (101) noted that, 20 min after injection of ET into the skin of a newborn mouse, the intercellular vesicles had widened and the oval profiles had largely disappeared. After 25 min, when the Nikolsky sign was positive, desmosomes began to split and separate. After 150 min, cleft formation (Fig. 4) was apparent within the stratum granulosum but the granular cells were unaffected. All desmosomes along the cleft were split dramatically, with half of each desmosome remaining with its parent cell. Desmosomal splitting was strictly localized to the stratum granulosum. Cell separation occurred without significant cytolysis. To explain the selective induction of desmosomal splitting, Lillibridge et al. (101) suggested that the intercellular "bubbles" might contain an enzyme or proenzyme that was activated by ET. The activated enzyme might then cause splitting of the nearest desmosomes, and as diffusion of the enzyme proceeded, desmosomes then became split in adjacent cells.

However, an ultrastructural study by Elias et al. (44) provided evidence that challenged the theory of Lillibridge and co-workers (101). After administration of ET to neonatal mice, histological sections showed that, during early events, desmosomes often did not separate, while interdesmosomal regions ballooned. At other sites separation appeared to occur simultaneously along entire adjacent cell surfaces. Cells in the region of the epidermal split showed neither cytolysis nor hydrolysis of the mucopolysaccharide of the glycocalyx (intercellular cement). No unique epidermal structures were ever noticed at the interface of a cleavage plane. The most significant conclusion of this work is that extracellular components along interdesmosomal regions seemed to be initially affected by the toxin. Thus, contrary to the theory proposed by Lillibridge et al. (101), desmosomes did not appear to be the primary target of ET action. Ultrastructural studies by McLay et al. (116) also indicated that the splitting of desmosomes was probably a secondary rather than a primary event during intraepidermal cleavage. These investigators challenged 3-day-old mice with highly purified ET and then observed a long lag period followed by a rapid formation of distended gaps between cells along a horizontal cleavage plane. Contact between cell layers, at this time, was only maintained at desmosomal junctions. Thus, the ET appeared to be initially affecting intercellular cementing components other than desmosomes. The disruption of these cementing components was followed by the rush of fluid into the newly formed spaces between cells. This was believed to place abnormal stress on desmosomes which, in turn, split. In any event, it is clear that ET affects the normal forces of cell adhesion between susceptible cells of the stratum granulosum.

The electron microscope studies of McLay et al. (116) confirmed those of Lillibridge et al. (101) in relation to the disappearance of bubbles or intercellular vesicles in the ET-treated skin of neonatal mice. However, the former group of workers failed to obtain convincing evidence for the release of proteases from these vesicles to induce epidermal splitting as proposed by Lillibridge et al. (101). McLay et al. (116) observed that Trasylol, a tissue protease inhibitor, did not protect against epidermal splitting in neonatal mice after it was administered subcutaneously at different times in single and multiple doses before and after injections of ET. In similar studies, Weupper et al. (211) and Elias et al. (45) were unable to prevent ET action by pretreatment of susceptible tissues with a variety of protease inhibitors. However, it should be kept

in mind that morphological observation of the effects of protease inhibitors in vivo may not be the most reliable method to rule out proteolysis as one of the events leading to exfoliation. In a system defined for in vitro exfoliation, it was shown that exfoliation occurs optimally at 37°C and ceases at 4°C (45). Cleavage occurred over a pH range of 5 to 9 but not at or below a pH of 4. Although treatment with cycloheximide or puromycin produced a 90 to 95% decrease in epidermal protein synthesis in vitro, exfoliation was not inhibited.

A good clue to the action of ET would be the discovery of any existing epidermal binding sites. Such binding sites should be highly accessible to ET in neonatal mice and human infants. However, recent results by Baker et al. (13) have revealed that radioiodinated ET did not bind to erythrocytes, leukocytes, trypsin-dispersed keratinocytes, heat-separated epidermises, or whole newborn mouse skin.

Another series of experiments, designed to identify surface sites that interact with the toxin, were run by Elias et al. (44). These investigators observed that ET neither removed nor interfered with human leukocyte antigens on lymphocyte surfaces. The toxin did not affect the stain on the surface of ruthenium red-stained keratinocytes, which indicated that the toxin did not remove stainable surface acid mucopolysaccharide in significant quantities. In other results it was shown that ET neither altered pemphigus antigen nor interfered with binding of pemphigus antibody. Pemphigus is a bullous disease. Antibodies from patients with this disease attach to sites within the intercellular space of stratified squamous epithelia. These sites include areas affected by ET. This indicated that the pemphigus antigen might have been related to the ET receptor site. Although more sophisticated experiments are warranted, the existing evidence suggests that the mechanism of action of ET might not involve direct interaction with cell surfaces.

In summary, ultrastructural studies have clearly indicated that the action of ET perturbs the forces of adhesion between susceptible cells of the stratum granulosum. However, the events associated with this action are merely open to speculation. All attempts to find a specific surface receptor or target site for ET have failed. It is possible that ET could enzymatically degrade an intercellular cementing component or induce an enzyme to accomplish this task. The toxin could mediate conformational changes by nonenzymatic interaction with intercellular adhesion substances or act as an inhibitor of intercellular binding factors. It is also possible that the toxin might enhance the secretion of tissue fluid into intercellular spaces of the stratum granulosum that might in turn apply enough stress to split cell layers. It is hoped that more extensive and sophisticated studies at the molecular level will be performed in the near future to test the veracity of the above suggestions.

Genetic Regulation

Melish et al. (121) speculated that ET synthesis might be under the control of a bacteriophage. Bacteriophage regulate toxin production in both *Corynebacterium diphtheriae* and group A streptococci. Nearly all staphylococci are lysogenic, and it was reported that the ability to produce alpha-toxin was conferred upon certain nontoxigenic staphylococcal strains by lysogenization (20). Rogolsky et al. (144) observed that 10 out of 12 ET phage group 2 Tox^+ strains harbored temperate phage that were absent from all Tox phage group 2 strains examined. However, when nontoxigenic strains were made lysogenic with phage preparations from toxin-forming cultures, the lysogens never gained the capability of producing ET. In other experiments, phage were eliminated from Tox^+ strains after treatment with mitomycin C and ethidium bromide (EB), but no distinct correlation between phage loss and the ability to form toxin was ever noticed (144). During the course of the phage-curing experiments, an extremely critical observation was made. Treated cells of strain UT0001, from which temperate phage was not eliminated, lost the ability to synthesize ET. The connection between the use of EB for phage curing and the documented evidence for the ability of EB to eliminate plasmids was realized.

Many staphylococcal strains carry R plasmids which usually house one or more genetic determinants for resistance to inorganic ions or to antibiotics (141). Staphylococcal plasmids can control the synthesis of bacteriocins (141, 145, 146, 178). There is strong evidence to indicate that a staphylococcal plasmid also controls the production of enterotoxin B (153).

To show that an extrachromosomal genetic determinant for ET synthesis existed, 12 Tox^+ strains were analyzed. Experiments were performed with these strains to (i) measure the spontaneous rate of loss of the ability to synthesize ET, (ii) show the effect of chemical agents on enhancing this rate of loss, (iii) show the effect of growth at elevated temperatures on the rate of loss, (iv) detect incidence of coordinate loss of other genetic markers, and (v) isolate a plasmid for ET synthesis. The results of these experiments indicated that extrachromosomal genetic factors regulated ET synthesis in 2 out

of 12 strains examined (144, 177, 178).

Strains UT0001 and UT0007 lost the ability to make ET after growth either in a final concentration of 0.003% SDS, in 6×10^{-6} M EB, or at 44°C (144, 177). Growth at 44°C, which resulted in an elimination frequency of 98% after 8.5 h of growth, was more effective than growth in either EB or SDS in eliminating the capability of the Tox^+ strain to make ET (144). Strain UT0007 also had a high spontaneous rate of loss of the genetic determinant for ET synthesis. Such elimination experiments are valid only if it can be shown that preexisting Tox^- variants were not selected during treatment of the Tox^+ cultures. The early appearance of Tox^- cells and subsequent rapid conversion to the Tox^- state during growth of strains UT0001 and UT0007 in either EB or SDS at 44°C suggested the elimination of an extrachromosomal determinant for ET synthesis and not the selection of a preexisting Tox^- variant (144). The appearance of ET-negative variants during growth of strain UT0007 at 44°C is shown in Fig. 5. ET-negative variants were initially detected after 3 h of growth at 44°C. They composed approximately 98% of the total population by 8.5 h. When strain UT0007, a Tox^- heat-cured substrain of UT0007, and a third Tox^+ UT0007 substrain that was isolated after 18 h of growth at 44°C were grown separately at 44°C, the Tox^+ strains and not the Tox^- strain had a growth advantage at the elevated temperature.

Although strain UT0007 is a β-lactamase producer, the gene for this enzyme was not coeliminated with the gene for ET synthesis. It appears that genetic determinants for penicillinase synthesis in phage group 2 staphylococci are chromosomal (146, 178). One of these determinants has been recently mapped next to a gene for isoleucine-valine biosynthesis on a phage group 2 staphylococcal chromosome (unpublished results, this laboratory). An effort was made to find other genes on the plasmid for ET synthesis. Warren et al. (177) discovered that, after growth of the UT0007 strain either in EB or at 44°C, the Tox^+ marker and the gene for a specific staphylococcin ($BacR_1$) were always jointly eliminated. This indicated that genes for these two traits occupied the same plasmid. It was later shown by Rogolsky and Wiley (145) that electrofocused preparations of $BacR_1$ have no ET activity and that electrofocused preparations of ET have no $BacR_1$ activity. This indicates that the two products are distinct. The bacteriocin was stable over a wide pH range, and thermoresistant and bactericidal to the same and related species without being autoinhibitory. Like other staphylococcins, $BacR_1$ had a broad spectrum of activity against gram-positive

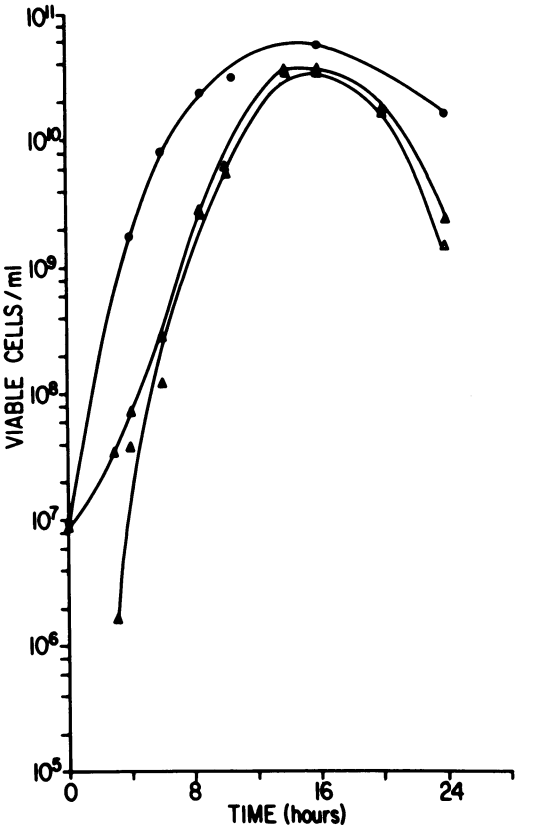

FIG. 5. *Appearance of ET-negative variants* (△) *during growth of strain UT0007 at 44°C* (▲). *Growth of strain UT0007 at 37°C* (●). *(Reproduced from reference 144.)*

bacteria (145) and was recently discovered to be also bactericidal to penicillin-resistant strains of *Neisseria gonorrhoeae* (128).

Although the data from our laboratory indicated that the genes for ET and $BacR_1$ synthesis were extrachromosomal, the identification of plasmid genes based solely on their elimination by chemical agents and by elevated temperatures suffers serious limitations (159). A gene for methicillin resistance can be eliminated from different staphylococcal strains after growth in either SDS, EB (153), or acriflavine (40), but no plasmid DNA associated with methicillin resistance has ever been detected (153, 159). Kuhl et al. (96) have provided evidence to show that methicillin resistance in *S. aureus* is governed by a single chromosomal locus, but they do not exclude the possibility that this locus can behave as a transposable element which accounts for its ambiguous association with plasmids. Therefore, definitive proof for a genetic trait residing on extrachromosomal deoxyribonucleic acid (DNA) is dependent upon distinct association of

this trait with plasmid DNA that is detected and characterized by standard procedures. Such procedures include agarose gel electrophoresis (122), dye-buoyant density gradient centrifugation, and neutral sucrose velocity centrifugation (146, 178). To accomplish this end, cell lysates prepared specifically for the isolation of plasmid DNA were taken from strain UT0007 and strain UT0100, which is a substrain of UT0007 cured of the ability to make ET and $BacR_1$ after growth in EB (178). The DNA preparations were then cosedimented to equilibrium after centrifugation through a CsCl-EB dye-buoyant density gradient. A dense band of DNA corresponding to extrachromosomal DNA was found in strain UT0007 (Tox^+ Bac^+) but not in strain UT0100 (Tox^- Bac^-) (Fig. 6). These data provided strong evidence to indicate that the genetic locus for ET synthesis in strain UT0007 resided on a plasmid. Similar evidence was used to show that the genetic determinant for ET synthesis in strain UT0001 was also plasmid borne.

To determine the number of molecular species of plasmid DNA in strain UT0007, the dense band of DNA from the dye-buoyant density gradient (Fig. 6) was isolated and centrifuged through a 5 to 20% neutral sucrose velocity gradient. Two species of DNA molecules with sedimentation coefficients of 56 and 38S were observed on the sucrose gradient (178). However, when the same plasmid DNA was isolated by the gentle cleared lysate technique (130) and directly analyzed on neutral sucrose velocity gradients, only the 56S species was seen (178). A molecular weight of 29,000,000 was calculated for the 56S plasmid (R. Warren, M. Rogolsky, and B. Wiley, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, H95, p. 112). Rosenblum and Tyrone (148) confirmed the presence of the 56S plasmid in strain UT0007 but found it to have a molecular weight of 26,000,000. Further investigation revealed that the plasmid for ET synthesis spontaneously converted from its native covalently closed circular 56S state to an open circular 38S form during storage (178). Conversion of the virulence plasmid to the open circular form was enhanced after interaction with either SDS, Pronase, EB, or alkali, but not after incubation with ribonuclease A or at 60°C for 15 min (Warren et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, H95, 112). Therefore, any procedure requiring the use of SDS or EB would set up conditions to promote conversion of the covalently closed circular to open state. ET plasmid DNA isolated by the SDS-NaCl procedure, but not by the cleared lysate procedure, consistently yielded the 56S and 38S species after direct, immediate analysis on neutral sucrose velocity gradients (178).

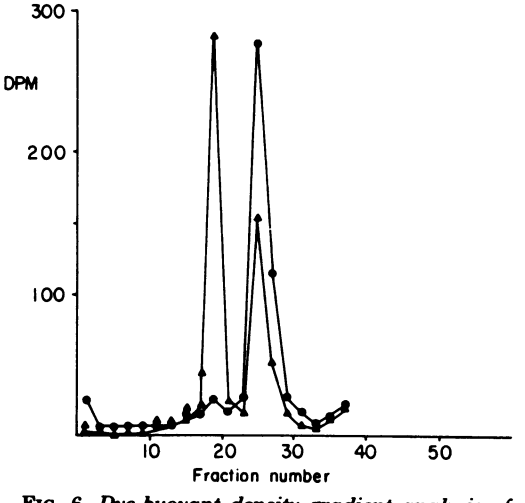

FIG. 6. *Dye-buoyant density gradient analysis of a [methyl-^{3}H]thymidine-labeled DNA preparation from strain UT007 (▲) and a [methyl-^{14}C]thymidine-labeled DNA preparation from strain UT0100 (●). Recovery of added radioactivity from the material layered on the gradient was greater than 90%. Strain UT0100, which lacks the heavy peak of plasmid DNA, was a substrain of strain UT0007 that was cured of the ability to synthesize ET. (Reproduced from reference 178.)*

The conversion of plasmids from a covalently closed circular to the open circular relaxed form after treatment with SDS or Pronase has also been described for certain *Escherichia coli* colicin plasmids (ColE1, ColE2, ColE3, ColV2, and Col1b), conjugative plasmid (F1), and R plasmids (R64, R28K, and R6K) (29, 62, 97). The ColE1 complex could be relaxed by EB (103). Such plasmids have been designated as relaxation complexes. After relaxation of the ColE1 complex, a 60,000-molecular-weight protein remained tightly bound to the nicked strand of open circular DNA (62). Specific endonucleases degraded the nicked strand from the 3′ end, but not the 5′ end, of an SDS-relaxed complex. However, after Pronase treatment, the 5′ end of the nicked strand was hydrolyzed by 5′ to 3′ T7 exonuclease which indicated that the bound protein was located at or near the 5′ end of the nicked strand. The protein component of the complex was believed to function as either a linker to restrain a specific single-strand nick or as a repressed endonuclease which could be activated after exposure to Pronase or SDS (103).

The ET plasmid was the first apparent relaxation complex observed in *S. aureus*. More recently, it was shown that six staphylococcal R plasmids behaved as relaxation complexes (129). Although there has not been any direct demonstration of protein bound to any of the staphy-

lococcal plasmid complexes, the indirect evidence for the protein, shown by Pronase- and SDS-induced relaxation, indicates that they are protein-DNA relaxation complexes. It is assumed that the staphylococcal complexes are similar to those found in *E. coli*. Both enteric and staphylococcal complexes show variability from plasmid to plasmid especially with respect to their stability and their relaxation after incubation at 60°C. The relaxation properties of the ET plasmid are similar to those reported for the *E. coli* R28K and ColE2 plasmids which also do not relax after incubation at 60°C (97). However, the staphylococcal, but not the enteric, molecule remains sensitive to Pronase and SDS after heat treatment (97, 146). This indicates that, if a protein is bound to the ET plasmid, it is not an inactive endonuclease. Such an enzyme would probably be denatured after exposure to high temperature which would make it nonresponsive to activation by Pronase or SDS.

In summary, it has been observed that phage group 2 staphylococci can house a plasmid with genes for ET and bacteriocin synthesis. The plasmid has an approximate molecular weight of 29,000,000 and a sedimentation coefficient of 56S. The 56S molecule appears to be a protein-DNA relaxation complex that relaxes after exposure to EB, SDS, and Pronase but not after incubation at 60°C.

It has already been mentioned that only 2 (UT0007 and UT0001) of 12 ET-producing strains could be cured for the ability to make toxin after elimination of a 56S protein-DNA relaxation complex (144, 178). Why cannot the remaining 10 strains be cured? Explanations considered were that the uncurable strains (i) harbored a plasmid that resisted standard curing procedures, (ii) contained chromosomal genes for ET synthesis, or (iii) contained both chromosomal and extrachromosomal genes for ET synthesis. If the last hypothesis were correct, specific strains could be cured of the virulence plasmid and still make toxin by way of chromosomal determinants. To test this hypothesis, strain UT0002 (Tox^+ Bac^+) and strain UT0003 (Tox^+ Bac^-) were grown at 44°C under the conditions used to coeliminate the genes for ET and $BacR_1$ synthesis in strains UT0007. The extracellular supernatant fluids of a number of treated substrains and the two parent strains were then assayed for ET activity after growth of these strains in heart infusion broth. The results of these assays indicated that a high percentage of the treated UT0002 and UT0003 substrains tested showed twofold and ninefold reductions in ET activity, respectively (146). The substrains with reduced ET activity showed normal in vitro growth patterns. In strain UT0002, the twofold loss in ET activity was always accompanied by complete loss of $BacR_1$ activity. Strain UT0003 was defective for $BacR_1$ production. Kapral (82) reported that the amount of toxin reduction in two of the treated substrains was much greater than that reported by Rogolsky et al. (146).

To be certain that reduced toxin production was directly related to plasmid loss, strains UT0002 and UT0003 and their substrains with reduced ET activity were analyzed for extrachromosomal DNA. When cleared lysates from strain UT0002 and one of its substrains (UT0002-19) with reduced ET activity were mixed and centrifuged through a neutral sucrose gradient, a 56S plasmid was present in the lysate coming from strain UT0002, but absent from the lysate prepared from the UT0002-19 strain (Fig. 7A) (146). Similarly, when mixed cleared lysates from strain UT0003 and one of its substrains with reduced ET activity were cosedimented through neutral sucrose, DNA corresponding to the ET plasmid was identified in the former but not the latter strain (Fig. 7B) (146). These observations indicated that partial reduction of ET activity in strain UT0002-19 and in the substrain of UT0003 was directly related to loss of the ET plasmid in these strains. The ET plasmid found in strains UT0002 and UT0003 was believed to be physically identical to the one described for strains UT0001 and UT0007. All of these plasmids had the same molecular weight and sedimentation coefficient and relaxed after treatment with EB, SDS, and Pronase, but not after incubation at 60°C for 15 min.

In addition to detecting the 56S virulence plasmid in strains UT0002 and UT0003, a second 21S plasmid was found (Fig. 7) (146). Since substrains of UT0002 and UT0003 which were cured of the 56S plasmid still produced ET, it was possible that the 21S plasmid contained a genetic determinant for ET synthesis. All attempts to cure the 21S plasmid from the two Tox^+ strains failed. Rogolsky et al. (144) had previously reported that, after mitomycin C induction of strain UT0003, a phase lysate could be secured that was capable of transducing an extrachromosomal gene for cadmium resistance into the phage group 2 UT0017 recipient. To determine whether the 21S plasmid was carrying a gene for cadmium resistance, phage lysates were prepared from strains UT0002 and UT0003 after induction with mitomycin C, and the cadmium resistance marker carried by these strains was transduced into the cadmium-sensitive, ET-negative, plasmidless strain UT0017 recipient (146). An analysis of cleared lysates from donor,

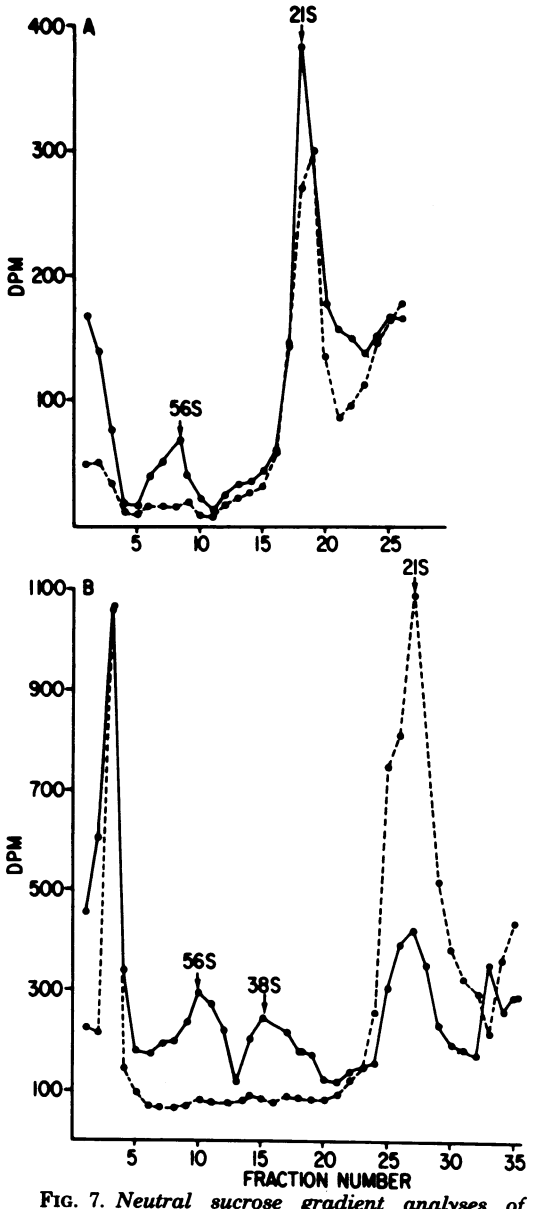

FIG. 7. *Neutral sucrose gradient analyses of mixed cleared lysates of (A) [methyl-^{3}H]thymidine-labeled DNA from strain UT0002 (—) and [methyl-^{14}C]thymidine-labeled DNA from strain UT0002-19 (----), and (B) [methyl-^{3}H]thymidine-labeled DNA from strain UT0003 (—) and [methyl-^{14}C]thymidine-labeled DNA from strain UT0003-4 (----). Recovery of added radioactivity from the material layered on the gradients was greater than 90%. Strains UT0002-19 and UT0003-4 are substrains of UT0002 and UT0003, respectively. These substrains were cured of the 56S plasmid but still contained chromosomal loci for ET synthesis. Therefore, strains UT0002-19 and UT0003-4 make reduced amounts of ET compared to their parent strains. The 38S peak represents the open circular form of the 56 plasmid. The 21S peak, which appears in the DNA of all four strains, represents a plasmid with a gene for cadmium resistance. (Reproduced from reference 146.)*

recipient, and recombinant strains on neutral sucrose gradients distinctly showed that cadmium resistance was associated with transfer of the 21S plasmid. Cadmium-resistant recombinants never received a genetic determinant for ET synthesis. This indicated that the residual toxin made by the 56S plasmid-cured substrains of UT0002 and UT0003 was controlled by the chromosome, and not by the 21S plasmid.

In summary, two phage group 2 staphylococcal strains were shown to contain both chromosomal and extrachromosomal genes for ET synthesis. The plasmid for ET synthesis in these strains was indistinguishable from the one found in strains which had only the extrachromosomal determinant for ET synthesis.

If naturally occurring phage group 2 strains exist with either plasmid control or both plasmid and chromosomal control of ET synthesis, it seems likely that naturally occurring strains would also exist with only chromosomal determinants for the toxin. The first report of such strains was made by Keyhani et al. (86). Two plasmidless phage group 2 strains that produced neither detectable ET by the standard assay procedure nor SSSS in the animal model were found to make minute quantities of toxin after their culture supernatant fluids were concentrated 20-fold and inoculated into newborn mice. In a later study, Rosenblum and Tyrone (148) reported that two Tox$^+$ phage group 2 strains, isolated from patients with SSSS, used only chromosomal determinants to regulate ET synthesis. These strains were referred to as TG and ER 201. Strain TG was found not to contain any plasmid DNA of a covalently closed circular or open circular nature after analyses on both dye-buoyant density gradients and 5 to 20% neutral sucrose gradients. Strain ER 201 contained a 56S plasmid similar to that observed in strains UT0007 and UT0002. However, elimination of the plasmid after growth of strain ER 201 at 43°C had no apparent effect on the toxigenicity of strain ER 201, as was measured by the direct inoculation of the parent and plasmid-cured substrain into newborn mice. Unfortunately, assays for ET synthesis were not carried out for strain ER 201 and its plasmid-cured substrain. Such assays might have shown that lower amounts of toxin were actually made by the cured strain similar to that seen for strain UT0002-19 (146). If such assays were performed and toxin amounts produced were indeed similar for the

plasmid and plasmid-cured substrains, one could speculate that the proposed plasmid gene for ET synthesis was defective. In a similar manner, it was shown that the 56S virulence plasmid for ET synthesis carried by strain UT0003 was defective for $BacR_1$ synthesis (146). Thus, it appears evident that all ET production is chromosomally determined in the plasmidless TG strain, but the evidence is less convincing for a like situation in strain ER 201.

It has been established that phage group 2 strains of *S. aureus* make two antigenically distinct types of ET and that control of ET synthesis in these strains can be either chromosomal, extrachromosomal, or a combination of both. Therefore, it would be of interest to identify the genetic determinant responsible for governing a specific molecular species of ET. To find out whether the extrachromosomal and chromosomal genetic determinants for ET synthesis were composed of the same or different genes, an attempt was made to determine if the nucleotide sequences of these two genetic loci shared homology. DNA-DNA hybridization experiments according to a modified procedure of Denhardt (35) were run between purified ET plasmid DNA and chromosomal DNA from strain UT0002-19 cured of the ET plasmid (183; unpublished results, this laboratory). Hybridization between two ET plasmid DNA preparations and two similar staphylococcal chromosomal DNA preparations were run as adjunct controls. The results of these experiments indicated that DNA from the ET plasmid did not share any detectable homology with the strain UT0002-19 chromosome. We interpreted these results to mean that the plasmid and chromosomal determinants for toxin synthesis had different functional nucleotide sequences.

The next logical step was to isolate the gene products made by the different genetic loci for ET synthesis. ET was purified from strains UT0002, UT0002-19, and UT0007 after isoelectric focusing on glycerol density gradients (185). Crude preparations derived from dialyzed $(NH_4)_2SO_4$-precipitated ET were also examined. Both the crude and electrofocused, purified preparations of toxin were loaded onto 12.5% SDS-polyacrylamide slab gels, and electrophoresis was carried out until the dye marker front reached the bottom of the gel. The gels were then stained with Coomassie brilliant blue. The results of these ET electrophoresis studies by Wiley and Rogolsky (185) are shown in Fig. 8. Channel 1 contained electrofocused toxin made by strain UT0002 and shows two components. Thus, a strain having both chromosomal and extrachromosomal genes for ET synthesis makes two distinct components of ET. Therefore, a strain with only extrachromosomal control of ET synthesis would be expected to yield only one of the two components. The validity of this assumption can be seen by observing channel 2, which contains electrofocused toxin from strain UT0007 and shows only one band. Channel 3 contains electrofocused ET prepared from strain UT0002-19 which was cured of the 56S virulence plasmid. This channel shows one major band that directly aligns with the top band in channel 1; this suggests that these two bands represent the ET product (ET A) governed by the chromosomal determinant. The band in channel 2 that directly aligns with the bottom band from channel 1 and that is missing from channel 3 is believed to be the ET product (ET B) regulated by the virulence plasmid. As mentioned above, ET A was heat stable and ET B was heat labile. Table 3 summarizes the data derived from the electrophoresis experiments. To determine whether the two gene products were antigeni-

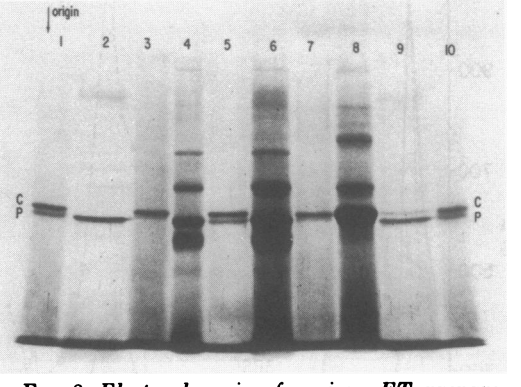

FIG. 8. *Electrophoresis of various ET preparations on a 12.5% SDS-polyacrylamide slab gel. The gel channels were loaded with either 70 to 100 μg of purified, electrofocused ET protein or 375 μg of partially purified toxin preparations (crude) obtained after $(NH_4)_2SO_4$ precipitation. Electrophoresis was carried out with constant voltage and a current of 35 mA until the front of the dye marker reached the bottom of the gel. C marks the bands of ET regulated by a chromosomal genetic determinant, and P marks the bands of ET regulated by a genetic determinant on the 56S virulence plasmid. (1) Electrofocused ET from strain UT0002; (2) electrofocused ET from strain UT0007; (3) electrofocused ET from strain UT0002-19; (4) crude preparation of ET from strain UT0007; (5) electrofocused ET from strain UT0002; (6) crude preparation of ET from strain UT0007; (7) electrofocused ET from strain UT0002-19; (8) crude preparation of ET from strain UT0002-19; (9) electrofocused ET from strain UT0002-19 mixed with electrofocused ET from strain UT0007; (10) electrofocused ET from strain UT0002. (Photograph provided by Bill B. Wiley and reproduced from reference 185.)*

cally distinct, Ouchterlony double-diffusion tests were carried out with antisera against electrofocused preparations of ET from strains UT0007, UT0002, and UT0002-19 (outer wells) and purified preparations of ET A and ET B (central well) (185). The antiserum made against the ET from strain UT0002 produced a double precipitin line after interaction with the purified ET mixture. The outer precipitin line formed a reaction of identity with the line made by the antisera prepared against strain UT0001-19. Therefore, the outer precipitin lines were believed to represent interaction between ET A and ET A antitoxin. The inner precipitin line made by the antisera against ET from strain UT0002 formed a reaction of identity with the line made by the antiserum against strain UT0007 ET and was believed to represent interaction between ET B and ET B antitoxin.

In summary, the chromosomal and extrachromosomal genetic determinants for ET synthesis in phage group 2 staphylococci represent different genes. Each genetic locus produces a distinct molecular species of ET. The chromosomal determinant governs the synthesis of ET A, and the extrachromosomal determinant governs the synthesis of ET B. ET A and ET B are serologically distinct proteins.

By means of DNA-mediated transformation, three genetic linkage groups were defined on the chromosome of staphylococcal phage group 3 strain 8325 (136; P. A. Pattee, S. A. Kuhl, D. R. Brown, N. J. Rasmussen, R. J. Johnson, and E. C. Swanson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, H94, p. 119). These studies made a number of genetic markers available for strain 8325. However, the strain 8325 genetic markers could not be utilized for mapping a phage group 2 genetic determinant for ET synthesis since intergroup transformations were found to be routinely unsuccessful (111). Either inactivation of a recipient's restriction endonucleases or the use of restriction enzyme-deficient mutant recipients prevented intergroup transformation (S. Martin and M. Rogolsky, unpublished results). This indicated that donor and recipient strains for transformation studies designed to map a gene for ET synthesis by using phage group 3 donor and recipient strains had to be confined to the same phage group. Attempts to map a gene for ET synthesis by using phage group 3 donor and recipient strains has not been reported. Thompson and Pattee (171) discovered that phage 80 alpha could be used to promote transformation in certain phage group 2 propagating strains. By using 80 alpha as a helper phage in the presence of Ca^{2+}, a transformation regimen was developed to detect linkage between a chromosomal gene for ET synthesis from phage group 2 strain UT0002-19 and either an auxotrophic or antibiotic resistance marker on the chromosome of the phage group 2 Tox^- recipient strain UT0017 (S. M. Martin, M. Alsup, B. B. Wiley and M. Rogolsky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, H96, p. 120). Three distinct genetic linkage groups were identified on the chromosome of strain UT0017 (111). However, a *tox*$^+$ trait for ET synthesis was associated neither with markers that composed the three genetic linkage groups nor with any one of a number of other auxotrophic and antibiotic resistance markers on the strain UT0017 chromosome (unpublished results, this laboratory).

If multiple unlinked genes for ET synthesis exist, cotransformation of the *tox*$^+$ genotype and a linked chromosomal marker might not be possible. Therefore, donor DNA from the ET-negative UT0017 strain was transformed into a Tox^+ UT0002-19 recipient strain to detect linkage between a *tox* marker and either a trait for amino acid biosynthesis or a trait for antibiotic resistance (S. Shoham, S. M. Martin, B. B. Wiley, and M. Rogolsky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, H97, p. 120). A *tox* trait could not be linked to 12 different markers on the strain UT0002-19 chromosome. In summary, a regimen for DNA-mediated transformation has been developed for phage group 2 staphylococci. Although a larger number of genetic markers have been isolated for phage group 2 strains, none of these markers cotransformed with a chromosomal locus for ET synthesis.

TABLE 3. *Association of the genetic determinants for ET synthesis with their corresponding toxin products*

Strain[a]	ET type produced	Location of the corresponding gene(s) for ET synthesis
UT0002	A	Chromosomal
	B	Extrachromosomal
UT0007	B	Extrachromosomal
UT0002-19	A	Chromosomal

[a] All strains are phage group 2.

ALPHA-TOXIN

Overview

Since the early classical studies of Burnet (23), alpha-toxin has been the most intensely studied staphylococcal toxin. Alpha-toxin has always been considered to play a significant role in the pathogenesis of staphylococci, but its exact role has never been defined. Its main significance in pathogenicity is that of producing tissue damage after the establishment of a focus of infection. It

is sometimes referred to as a hemolysin by virtue of its exquisite hemolytic effect on rabbit erythrocytes. Aside from being hemolytic, alpha-toxin is cytotoxic and cytolytic to a wide variety of cell types. It is also dermonecrotic and neurotoxic and kills experimental animals. Therefore, it has been suggested that the toxin be called a membrane-damaging toxin (112) or a cytolysin (17), rather than a hemolysin, in order to use a term that provides a more accurate description of the toxin's biological activity (53).

As cited in the introduction, *S. aureus* makes four distinct membrane-damaging toxins (alpha-, beta-, gamma-, and delta-toxin), all of which exert profound effects on cell membranes. Since many strains of *S. aureus* produce all four membrane-damaging toxins (73), it is mandatory to remove trace amounts of the other membrane-damaging toxins from purified alpha-toxin preparations when studying the toxin's biological and physicochemical properties. Failures to meet the criteria for purity have caused numerous misconceptions regarding the mechanism of action of alpha-toxin. Thelestam et al. (170) observed that the biological effects of crude and purified alpha-toxin preparations are drastically different. It was believed that much of the activity of the crude preparation was due to delta-toxin. As little as 0.1 hemolytic unit of delta-toxin can cause significant leakage of human fibroblast cells grown in vitro (170). Alpha- and delta-toxin cause distinctly different structural changes on the surface of susceptible cells (19). An early study claimed that alpha-toxin consisted of two distinct components, alpha-1 and alpha-2 (127). However, later work clearly indicated that alpha-2 and delta-toxin were identical (42). Colacicco et al. (30) recently admitted that lysis of spheroplasts and protoplasts by an alpha-toxin preparation, observed at an earlier time, was probably due to traces of contamination by delta-toxin. The above findings clearly establish the need to use highly purified toxin preparations to redefine the biological properties of alpha-toxin. Numerous methods for the purification of alpha-toxin are available and have been reviewed by Arbuthnott (3), Jeljaszewicz (73) and Wiseman (190). Many of these separation procedures involve preparative electrophoresis and ion-exchange chromatography, since alpha-toxin has an approximate isoelectric point of 8.5, and the other cytolysins have an isoelectric point of approximately 9.5 (190). Cassidy and Harshman (26) utilized a novel procedure to obtain highly purified toxin by selectively eluting the toxin from columns of small glass beads followed by diethylaminoethyl-Sephadex chromatography.

Properties

Purified alpha-toxin is a protein. The amino acid composition of the protein has been defined (190). Histidine, arginine, and alanine have all been detected as N-terminal amino acids. The variation in the N-termini was believed to be due to nicking by proteases present in the crude toxin preparations. Estimates for the molecular weight of the protein vary from 26,000 to 39,000 (34, 190) and depend upon the methods used for isolation and determination of molecular weight. Variation in molecular weight due to strain differences is not a factor, since nearly all investigators used strain Wood 46 (190). Dalen (34) reported that alpha-toxin from strain Wood 46 consisted of 27,500- and 39,000-molecular-weight components that were both antigenically identical. The smaller isomer was believed to release a 10,000-dalton peptide after proteolytic cleavage. The peptide then formed a dimer with the 27,500 component to produce the 39,000-dalton isomer. It was suggested that autodigestion resulting from proteolytic activity associated with purified toxin was responsible for the splitting of the 27,500-dalton component. However, the observed changes could have resulted from interaction of the toxin with extraneous proteases during the isolation procedure. Pure toxin is unstable in solution and completely destroyed by freezing. It has a half-life of 3 days in buffer at 0°C (73). The toxin in its purified state is electrophoretically heterogeneous. This is evidenced by its separation into as many as four electrophoretic varieties that appear to be identical immunologically and in their biological effects (17, 117). The major component of the four electrophoretic variants had an isoelectric point of 8.5 and accounted for 80 to 90% of the total recovered hemolytic activity (117). The minor forms also had hemolytic activity and isoelectric points of approximately 6.3, 7.2, and 9.1. All four components were apparently interconvertible. Two electrophoretically distinguishable noninterconvertible forms of alpha-toxin, designated A and B, were isolated by Six and Harshman (154). They were considered to be "charge isomers" rather than "size isomers" and yielded 27 and 24 peptides, respectively, after hydrolysis with trypsin.

The sedimentation coefficient of alpha-toxin is approximately 3.0S (17, 190). However, the toxin can also exist in a 12S (or 16S) form. As first suggested by Lominski et al. (102), the 3S form aggregates or polymerizes into the 12S form. Approximately 8 to 15% of purified alpha-toxin preparation consists of the 12S component (3, 154). Purified preparations of 12S toxin are generally considered to be biologically inactive

(73, 190), but some isolates have been reported to have hemolytic activity (17, 54). In any event, treatment of 12S material with 8 M urea was accompanied by a substantial gain of hemolytic activity and the appearance of 3S protein that was indistinguishable from the native 3S cytolysin (6).

The 3S toxin was shown to consist of an amorphous, finely granular substance after examination under the electron microscope (54). Alternately, 12S toxin was composed of rings or doughnut-shaped structures with an outer diameter of 10 nm. Each doughnut-shaped structure contained a hexagonal array of six circularly arranged subunits, each of which measured 2.0 to 2.5 nm in diameter. McNiven and Arbuthnott (117) estimated the molecular weight of 12S toxin to be 170,000. This indicated that each of the six subunits is a 3S monomer with an approximate molecular weight of 28,000.

Alpha-toxin interacts with a wide variety of cell types and is associated with a wide spectrum of cytotoxic and pharmacological effects. These aspects of alpha-toxin activity are adequately and comprehensively reviewed elsewhere (3, 73, 74, 98, 161). Induced cellular effects by the toxin have generally been described in terms of morphological and physiological alteration. Electron microscopy has been a valuable tool for studying the morphological changes. The mode of toxin action at the molecular level is not completely understood. However, there is much evidence which indicates that the plasma membrane of sensitive cells is the likely target for the toxin. Therefore, the mechanism of action of alpha-toxin can best be interpreted by investigating how it alters membrane structure and function. Toxin-membrane interactions can be studied with either membranes of intact cells or with osmotically prepared, washed membrane preparations. However, toxins may react differently with free isolated membranes as compared to intact membranes. An important difference between these two systems is that free membranes have two surfaces exposed to the action of toxin.

The sensitivity of erythrocytes to lysis by alpha-toxin varies greatly. A concentration of alpha-toxin capable of completely lysing a population of rabbit erythrocytes will lyse less than 1% of a population of chicken, guinea pig, human, horse, or monkey erythrocytes (17, 73). Cells with low sensitivities might lack specific receptors for alpha-toxin or might differ in their relative distribution or composition of membrane protein and lipid compared to rabbit erythrocytes. In support of this hypothesis, Cassidy and Harshman (24) observed that the capacity of erythrocytes from different animal species to bind toxin correlated with cellular hemolytic sensitivity.

Since many early kinetic studies indicated that the rate of hemolysis was directly proportional to the concentration of alpha-toxin (53), it was thought that the toxin might function enzymatically. Wiseman and Caird (193, 194) provided evidence to indicate that alpha-toxin is secreted as an inactive protease which, after activation by a membrane-bound proteolytic enzyme, hydrolyzes structural membrane protein. Wiseman (190) further speculated that toxin interaction with membrane lipoprotein might trigger activation of the toxin by the protease activator. According to this theory, erythrocyte sensitivity would be directly related to the native proteolytic activity of a specific erythrocyte membrane. Wiseman and co-workers (193–195) observed that alpha-toxin-treated rabbit erythrocyte membranes did not release acid-soluble phosphorus but did yield nitrogen that increased with time. Nitrogen release from different erythrocyte membranes directly correlated with hemolytic sensitivity. Alpha-toxin was shown to be proteolytic in the presence of membranes and released 9% of the protein content from such membranes. Further results indicated that trypsin-activated alpha-toxin could hydrolyze tosyl arginine methylester (195). Dalen (34) also suggested that alpha-toxin might be proteolytic. Other workers were unable to corroborate that the mechanism of action of alpha-toxin included proteolysis. Freer et al. (55) found that rabbit erythrocyte stomata treated with alpha-toxin showed no alteration in polypeptide patterns and no reduction in sedimentable protein. They also observed that alpha-toxin-induced hemolysis could not be prevented by protease inhibitors. Arbuthnott et al. (8) noted that trypsin, chymotrypsin, Pronase, and subtilisin, but not alpha-toxin, released substantial amounts of protein from free erythrocyte membranes. Any association of alpha-toxin with enzymatic activity must take into account possible toxin contamination. Alpha-toxin preparations were observed to behave enzymatically in deesterifying cholesteryl esters in lipoprotein (66). However, other studies indicated that purified preparations of alpha-toxin could be contaminated with a cholesteryl esterase. This was evidenced by the separation of esterase and hemolysin activity after isoelectric focusing of a partially purified toxin preparation (113).

Hemolysis of erythrocytes by alpha-toxin involves initial binding of the toxin which results in the release of K^+ followed by the release of hemoglobin and lysis (17). Scanning and transmission electron microscopy indicated that toxin

binding initiated segmental separation of the erythrocyte membrane (87). Immunofluorescent studies by Klainer et al. (88) indicated that toxin was present on the surface of intact rabbit erythrocytes during the period of maximal hemolysis. This was followed by a decrease in fluorescence with time. Surprisingly, no fluorescence was detected during the prelytic period. Fluorescence of human erythrocytes occurred only when the toxin concentration was increased approximately 100 times over that used for rabbit erythrocytes. Cassidy and Harshman (24) found that ^{125}I-labeled alpha-toxin bound very rapidly to intact rabbit erythrocytes, during the prelytic stage, but only about 5% of the added toxin became bound. When relatively low concentrations of ^{125}I-labeled alpha-toxin were used, only transient binding occurred. However, 60 to 70% binding was obtained when relatively high concentrations of ^{14}C-labeled alpha-toxin were added to osmotically prepared erythrocyte ghosts (53). The binding of the ^{14}C-labeled toxin decreased dramatically as the ionic strength of the reaction mixture was increased to that of isotonic conditions (53).

Cassidy and Harshman (25) carried out extended studies on the binding of alpha-toxin to rabbit erythrocytes with highly purified toxin preparations obtained after adsorption chromatography on glass beads (26). The specificity of the binding reaction was determined by showing that native alpha-toxin and ^{127}I-labeled alpha-toxin block the binding of ^{125}I-labeled alpha-toxin to a specific receptor site in the erythrocyte membrane. Radioiodinated toxin was restricted in hemolytic action but not in binding. The binding of radioiodinated toxin to the erythrocyte was found to be irreversible, and, at toxin concentrations around 1.5 μg/ml, the toxin receptor sites became saturated. The rate and extent of binding were both temperature dependent. Other data indicated that binding of toxin, release of K^+ ions, and release of hemoglobin are separate sequential events in the hemolytic pathway. Although the lag period before the release of K^+ ions appeared to be independent of toxin concentration, the maximum rate of ion release was dependent on both toxin concentration and amount of toxin bound. This was interpreted as meaning that each bound alpha-toxin molecule initiates a focus of membrane disruption which leads to cytoplasmic leakage. Rabbit erythrocytes appeared to contain approximately 5×10^3 binding sites per cell compared to no detectable binding sites for human erythrocytes. Although the chemical nature of the binding site is still not known, Cassidy and Harshman (25) observed that the ability of toxin to bind to erythrocytes is lost after prolonged treatment of these cells with Pronase. This indicates that the membrane receptor site might be a protein or contain a protein component. However, Kato and Naiki (85) noted that *N*-acetylglucosamine containing ganglioside from human erythrocytes inhibited both hemolytic activity and binding of alpha-toxin to rabbit erythrocytes. Colacicco and Buckelew (31) also observed inhibition of hemolytic activity by gangliosides, but Wiseman (190) found evidence to show that the reaction between alpha-toxin and ganglioside was nonspecific.

In summary, there is controversy about whether alpha-toxin functions as a proteolytic enzyme to disrupt sensitive erythrocyte membranes. Although the proposed enzymatic mechanism should not be ruled out, more convincing evidence must be provided to substantiate its occurrence. It seems evident that binding of the toxin molecule to the membrane is required for the lysis of the intact erythrocyte, but there are conflicting data concerning the nature of the receptor site and the kinetics of binding. The use of varying experimental conditions might account for some of these conflicts. The degree of toxin-membrane interaction depends on specific conditions that include the ratio of toxin to membrane material, the ionic strength of the reaction mixture, and whether intact erythrocytes or isolated erythrocyte membranes are employed.

Staphylococcal alpha-toxin disrupts not only intact or isolated membranes of erythrocytes but also membranes of other types of mammalian cells which include platelets (19), hepatocytes (19), white blood cells (73), human diploid fibroblasts (170), HeLa cells (170), and Ehrlich ascites carcinoma cells (107). In contrast, bacterial membranes are unaffected by the toxin. No surface changes were seen by electron microscopy after interaction of bacterial membranes with alpha-toxin (19). This significant difference in response to alpha-toxin could be explained by the different chemical composition and distribution of lipids within the membranes of bacterial and mammalian cells.

A reliable technique to determine susceptibility of specific membranes to alpha-toxin is to demonstrate leakage from intact cells after exposure to toxin. K^+ ions, nicotinamide adenine dinucleotide, ^{32}P, and [^{35}S]methionine were all successfully employed as cytoplasmic markers to demonstrate leakage (107, 169). The data from these leakage experiments provided some insight into the kinetics of the toxin-membrane interaction. Thelstam and Möllby (169) compared the release of [^{3}H]uridine and the small-

molecular-weight, non-metabolizable ^{14}C-labeled α-aminoisobutyric acid markers from human diploid fibroblasts treated with highly purified alpha-toxin. Very little labeled nucleotide was released after 30 min of interaction, but leakage of the nucleotide gradually increased with time. No dramatic morphological changes, determined with the light microscope, accompanied leakage. The α-aminoisobutyric acid release assay was much more sensitive than the nucleotide release assay. A significant release of α-aminoisobutyric label occurred after 10 min of incubation and reached 75% after 30 min (167). The fibroblasts remained viable, and the light microscope revealed no morphological changes in cells releasing up to 100% of the leakage marker. Thelstam and Möllby (169) concluded from these studies that low concentrations of alpha-toxin were adequate to upset membrane permeability and that the release of cytoplasmic molecules was dependent upon toxin concentration. The membrane lesions produced by alpha-toxin were smaller than those induced by delta-toxin (168).

Incubation of 3S alpha-toxin with a variety of osmotically prepared natural mammalian cell membranes led to the formation of ring-shaped structures identified by electron microscopy (19). Similar ring structures were observed in erythrocyte membranes lysed by alpha-toxin (54). Treatment of the ring-laden membranes with 8 M urea (19) released a substantial amount of hemolytic activity, which indicated that the ring structures were the inactive 12S forms of alpha-toxin. The exact time of ring formation after exposure of mammalian cell membrane to toxin could not be determined. Electron photomicrographs showed that toxin-treated, freeze-etched rabbit platelet membranes were covered by a rectangular array of rings (19). Evidence from ultrastructural studies by Bernheimer et al. (19) further indicated that the rings penetrated the hydrophobic central plane of the membrane. Contrary to that seen by Bernheimer's group, Freer et al. (55) observed smooth depressions and plaques, but no ring structures, in freeze-etched photomicrographs of erythrocyte membranes exposed to the toxin. The plaques, similar to those made after treatment of erythrocyte membranes with phospholipase A or low concentrations of saponin (158), were thought to be caused by alternation of the fracture plane between adherent membrane material. Prolonged membrane-toxin interaction caused enhanced membrane perturbation which was manifested by a high incidence of cross fractures similar to that observed after treatment of the same membrane with high levels of saponin, lysolecithin, or SDS (158). There is no clear explanation for the different morphological surface features observed by Bernheimer et al. (19) and Freer et al. (55). However, it must be kept in mind that the effects of alpha-toxin on membranes are dependent upon purity of the toxin preparation and variables in the reaction mixture as mentioned above. In any event, the results of the freeze-etching studies indicate that alpha-toxin might be surface active and its mode of action might involve penetration and disruption of hydrophobic regions of cell membrane.

Further evidence for alpha-toxin being surface active was derived from studies on toxin interaction with isolated lipid components. Attention focused on the interaction of alpha-toxin with lipid components of natural membranes following the observation of Weissman et al. (180) that alpha-toxin could induce the release of marker molecules from liposomes. The reaction was inhibited by antisera prepared against alpha-toxin. Freer et al. (54) showed that alpha-toxin disrupted lipid spherules with the formation of ring structures characteristic of 12S alpha-toxin. The 12S ring polymers are formed upon the addition of 3S toxin to liposomes composed of many different individual lipids (19). This is indicative of the high surface activity of the toxin but further indicates that interaction of toxin and lipid induces polymerization of 3S to 12S toxin. Extended studies by Arbuthnott et al. (7) showed that polymerization could be induced by diglyceride, lecithin, cholesterol, or lysolecithin. No individual component was as effective as a mixed dispersion of lecithin, cholesterol, and dicetyl phosphate in producing polymerization. It was concluded that there was no specific lipid inducer, and interaction between toxin and natural membranes was dependent upon the location and distribution of specific lipids.

Buckelew and Colacicco (22) discovered that alpha-toxin forms a thick film on aqueous media that is capable of penetrating lipid monolayers. Rates of penetration were greatest with cholesterol and lowest with gangliosides. This work indicated that surface activity of alpha-toxin may contribute to its action on membranes. Studies with phospholipid model membrane systems have yielded many interesting results. However, the significance of these results in understanding the mode of action of alpha-toxin may be less than desired. Model membrane systems are very different from the natural membranes of susceptible cells. For example, Cassidy et al. (27) found that lipid spherules prepared from rabbit erythrocytes and lipid spherules prepared from human erythrocytes were equally sensitive to the action of alpha-toxin. This indi-

cates that the selective membrane-damaging effects of alpha-toxin involve something more than mere penetration of lipid bilayers. Thus, the pressing question of what makes rabbit erythrocytes 100 times more sensitive to alpha-toxin than human erythrocytes will probably not be answered by using the model membrane system.

Interrelation in Disease

Both warm- and cold-blooded animals are susceptible to cytotoxicity by alpha-toxin, and the degree of severity is dose dependent. The mean lethal dose of the toxin is approximately 30 μg/kg of body weight for adult mice injected intraperitoneally (3). Rabbits injected intravenously with a minimal lethal dose die after a few days (179). The major pathological finding was kidney necrosis usually accompanied by flaccid paralysis of the hind legs. Larger doses produced respiratory difficulty, intermittent muscular spasms, intravascular hemolysis, and death within minutes. After inoculation of ^{131}I-labeled alpha-toxin into rabbits, Jeljaszewicz et al. (76) determined that the toxin was distributed to nearly every body organ, but the greatest amounts were detected in the kidneys and lungs. Appreciable amounts of toxin were also detected in the brain. Extended studies with highly purified alpha-toxin by Edelwejn et al. (41) indicated that the primary lethal effect of the toxin in rabbits was triggered in the hypothalamus reticular system and in the visual-sensory region of the cerebral cortex. Death resulted from a rapid collapse of brain bioelectric activity.

Work in other laboratories indicated that the lethal target might be localized at a site other than the brain. Alpha-toxin caused constriction of coronary arteries and systolic arrest in perfused heart muscle from the rabbit, chicken, and cat (181). A decline in cardiac output was observed by Samanek and Zajic (150) in animals receiving alpha-toxin. These workers also noted the ability of the toxin to affect blood pressure that was presumed to be caused by disturbances within smooth muscle of the blood vessels. Alpha-toxin interacted with isolated smooth muscle which caused spastic spasm of fibers (74) and vasospasm of small blood vessels (166). The toxin can also promote spastic paralysis of the smooth muscle of guinea pig ileum (27). In summary, there appears to be no unified concept on the mechanism of lethal action for alpha-toxin, but the site for this action appears to be located in either the circulatory system or the central nervous system.

Genetic Regulation

Blair and Carr (20) suggested many years ago that alpha-toxin production might be under the control of a bacteriophage. However, Hendricks and Altenbern (69) could find no evidence to support this hypothesis. McClatchy and Rosenblum (114) carried out some important genetic analyses with a number of alpha-toxin-negative mutants they isolated from *S. aureus* 233. The mutants fell into two distinct groups, indicating that at least two genetic loci were involved in toxin production. Two-point reciprocal crosses by transduction between mutations within each group never produced alpha-hemolytic recombinants. Alternately, similar genetic crosses between mutations from different groups produced toxin-positive recombinants. Mutants from one group were always defective in both fibrinolytic and hemolytic activities, but these activities were subsequently shown to be associated with the same genetic locus. Since mutation within this locus resulted in a pleiotropic effect, a regulatory role for the locus was suggested. The mutants of the second group were always fibrinolytic positive and were believed to possess genetic defects within the same structural gene for alpha-toxin synthesis.

More recently, Brown and Pattee (Abstr. Annu. Meet. Amer. Soc. Microbiol. 1978, H95, p. 119) were able to map a genetic determinant for alpha-toxin production on the chromosome of staphylococcal phage group 3 strain 8325. Multifactorial crosses by DNA-mediated transformation established that the locus for toxin synthesis was positioned between a gene for purine biosynthesis and a gene for isoleucine-valine biosynthesis. As mentioned previously, three genetic linkage groups have been defined for the chromosome of strain 8325. The gene for alpha-toxin synthesis resided in linkage group 3.

Preliminary work by Witte (196) indicated that alpha-toxin can also be controlled by a genetic determinant that is extrachromosomal. The ability to make alpha-toxin was irreversibly lost at a high spontaneous rate in a number of non-phage group 2 *S. aureus* clinical isolates. The ability to produce alpha-toxin was lost from 5 out of 11 clinical isolates after growth in the presence of SDS. Additional evidence for the location of an extrachromosomal gene for alpha-toxin was indicated after application of the Arber test (2). This test is based on enhanced transduction frequencies of chromosomal but not extrachromosomal genetic determinants after irradiation of transducing phage with ultraviolet light. Ultraviolet irradiation of preparations of transducing phage was observed to enhance the frequency of transduction of the chromosomal trait for streptomycin resistance, but not the trait for alpha-toxin production. It is

hoped that future investigations will provide physical evidence of the plasmid with gene(s) for alpha-toxin production. The data from the studies of Witte (196) indicate that there is either more than one genetic determinant for alpha-toxin synthesis or that the gene for toxin production is associated with a transposon.

BETA-TOXIN

Overview

Beta-toxin has been recognized as an antigenically distinct staphylococcal product for more than 40 years. Production and purification of the toxin have been reviewed by Jeljaszewicz (73) and Wiseman (190). It is a protein of known amino acid composition (18). The protein has an approximate molecular weight of 30,000 (18, 124) and an approximate isoelectric point of 9.5 (190). Purified beta-toxin is heat labile, unstable in solution, and has a half-life of less than 1 day at either 4 or −20°C (174). However, it is stable after lyophilization. Purified toxin is cytotoxic to a wide variety of cell types, causing significant degradation of membrane sphingomyelin (189). It damages the plasma membranes of erythrocytes, leukocytes, and macrophages (74) and causes leakage of the small-molecular-weight α-aminoisobutyric acid label from human diploid fibroblasts (169). It is hemolytic and most active on ovine and bovine erythrocytes. The cytotoxic effects of beta-toxin are covered in detail in recent reviews by Jeljaszewicz (74) and Szmigielski et al. (161).

Properties

Beta-toxin (sphingomyelinase C) shares the distinction with alpha-toxin (phospholipase C) of *Clostridium perfrigens* type A in being the only membrane-damaging toxins with known enzymatic modes of action. Both toxins lyse sensitive cells by hydrolysis of membrane phospholipid. Doery et al. (37, 38) were the first to observe that beta-toxin hydrolyzed sphingomyelin. They identified the end products of hydrolysis to be *N*-acyl sphingosine and phosphorylcholine after thin-layer chromatography. Lysophosphatidlycholine is the only other phospholipid enzymatically degraded by beta-toxin (37). An Mg^{2+} requirement for beta-toxin activity has been firmly established (37, 61, 65, 108, 191). The sphingomyelinase activity of the toxin has been documented by a number of independent laboratories (18, 32, 104, 108, 124, 174, 191). Wiseman and Caird (191) were the first to make the important observation that sensitivity of different erythrocytes to beta-toxin was related to their sphingomyelin content.

A very interesting and significant study on the interaction of beta-toxin with intact erythrocytes was made by Colley et al. (32), whose work is summarized in Table 4. They observed that approximately 50% of the total sphingomyelin content of sheep and ox erythrocyte membranes could be hydrolyzed after interaction with beta-toxin. Sphingomyelin composed approximately 50% (wt/wt) of the total membrane phospholipid of these cells, meaning that about 25% of the total membrane phospholipid in the sheep and ox erythrocytes was degraded by the toxin. After enzymatic degradation, these erythrocytes are extremely fragile, but do not necessarily lyse when held at 37°C in the presence of Mg^{2+} (17). Although human erythrocytes are only slightly susceptible to beta-toxin action, they have approximately 26% (wt/wt) of their total membrane phospholipid in the form of sphingomyelin, of which approximately 77% is degraded after exposure to the toxin (Table 4). After enzymatic degradation, the membrane of the human erythrocyte shows much less damage than the membrane of the bovine erythrocyte, which makes the human erythrocyte more stable and less prone to lyse (18, 104). Therefore, it can be assumed that the amount of sphingomyelin degraded in a treated erythrocyte is not necessarily related to its susceptibility to lysis by the toxin.

At this point, it would be pertinent to ask what the factors that determine the degree of sphingomyelin degradation in a specific erythrocyte membrane would be. The data in Table 4 indicate that the sphingomyelin content of erythrocyte membranes is more efficiently hydrolyzed in the presence of relatively high concentrations of phosphatidylcholine. The location of the sphingomyelin in membrane could also determine its susceptibility to the toxin. There is strong evidence which indicates that phospholipids are not evenly distributed throughout the inner and outer layers of erythrocyte membranes (21, 28, 172, 213). Most of the total choline-containing phospholipids (sphingomyelin and phosphatidylcholine) are believed to be located in the exterior layers of the membrane, whereas most of the phosphatidylethanolamine and phosphatidylserine are believed to be located in the inner leaflet of the membrane (21, 28). This would indicate that toxin activity is limited to accessible regions of the outer part of the membrane. When beta-toxin interacted with bovine and human erythrocyte ghost membranes where accessibility to both sides of the membrane is possible, sphingomyelin hydrolysis was significantly enhanced but was never carried to completion (104). The degree of hydrolysis was 61 to 77% for bovine ghost membranes and 90% for human ghost membranes. The reason why a

TABLE 4. *Relationship between beta-toxin action and the phospholipid composition of erythrocyte membranes*[a]

Phospholipid component	% of component in total erythrocyte membrane phospholipid			
	Human	Swine	Ox	Sheep
Phosphatidylserine	14	18.4	20.7	16.8
Phosphatidylcholine	32.3	25.6	3.0	2.2
Sphingomyelin	26 (77)[b]	21.4 (75)	44.8 (50)	50 (48)
Phosphatidylethanolamine	27.5	34.5	31.5	31.1

[a] Derived from the data of Colley et al. (32).

[b] Figures in parentheses denote the percentages of sphingomyelin degraded by beta-toxin under optimal in vitro conditions. The toxin was interacted with intact erythrocytes.

portion of membrane sphingomyelin remains resistant to hydrolysis is not clear. It is possible that, even in free membrane preparations, not all the substrate is available to the enzyme. Another possibility might be that some sphingomyelin complexes with other membrane components rendering it unable to form an enzyme-substrate complex. It was also pointed out by Freer and Arbuthnott (53) that the overall surface charge and concentration of enzyme-dependent bound divalent cations would be expected to fall progressively with hydrolysis due to the release of the water-soluble polar moiety of sphingomyelin.

Incubation of small quantities of beta-toxin with washed sheep erythrocytes at 37°C in the presence of Mg^{2+} results in little or no lysis. However, if these treated erythrocytes are chilled at 4°C for a short time, rapid lysis of the cell population occurs (17). Bernheimer (17) suggested that extensive hydrolysis of the sphingomyelin content of intact erythrocytes by beta-toxin could induce thin lipid monolayers in regions of the membrane. The lipid monolayers were believed to be sufficiently stable at 37°C but not at lower temperatures where thermodynamic instability could lead to membrane monolayer collapse. The significant increase in lysis of beta-toxin-treated cells that is induced by a period of chilling after incubation at 37°C is a phenomenon known as hot-cold lysis.

The assay for hemolytic activity induced by beta-toxin is usually performed by incubating toxin with erythrocytes at 37°C in the presence of Mg^{2+} followed by a period of incubation at 4°C. Lysis of sensitive sheep erythrocytes can increase by as much as 10^8-fold when the incubation temperature of treated cells is changed from 37 to 4°C.

Degradation of a high percentage of membrane sphingomyelin followed by chilling does not necessarily result in hemolysis (32). This can be explained if the sphingomyelin in the cell membrane represents only a small part of the total phospholipid accessible to the toxin. Hydrolysis of sphingomyelin under these circumstances would probably result, therefore, in minimal disruption of the outer lipid membrane leaflet and little or no response to the hot-cold reaction. Alternately, the data in Table 4 indicate that beta-toxin could hydrolyze approximately half of the outer membrane leaflet of ox and sheep erythrocytes if all or nearly all of the sphingomyelin content of these cells were located in the outer region of the membrane. This would tend to indicate that beta-toxin can induce hemolysis only if it is capable of hydrolyzing approximately 50% of the phospholipids in the outer portion of the membrane.

The exact physicochemical changes that take place during hot-cold lysis have still not been adequately explained. As mentioned above, Bernheimer (17) suggested that hemolysis of sensitive cells below 10°C resulted from thermodynamic instability of hydrolyzed membranes. Meduski and Hochstein (118) suggested that hot-cold lysis is caused by changes that affect the choline residues of degraded sphingomyelin. Avigad (12) has suggested that hot-cold hemolysis occurs after a shift in the molecular reorganization of the residual intact membrane to allow for passage of hemoglobin. The temperature change was believed to affect the liquid crystalline state of the disrupted membrane directly. It was noted that temperatures below 17°C can cause significant thermotropic lipid clustering and lateral separation of rigid lipid particles.

Hemolysis of susceptible erythrocytes after exposure to beta-toxin can be induced by factors other than low temperature. These factors include centrifugation, osmotic shock, and rapid alteration of pH or NaCl concentration (188). Smyth et al. (156) showed that an effect resembling that of hot-cold lysis occurs after treatment of beta-toxin-digested erythrocytes with chelating agents such as ethylenediaminetetraacetic acid. Toxin-treated sheep but not human erythrocytes respond to chelator-induced hemolysis (124). This would indicate that divalent cations

stabilize beta-toxin-treated erythrocytes. Divalent cations are known to stabilize biological membranes (140), but little is known about the identity of these ions. The data of Smyth et al. (156) indicated that Mg^{2+} ions play some role in stabilizing sphingomyelin-depleted erythrocytes against lysis at 37°C. There appears to be a direct correlation between susceptibility of erythrocytes to hot-cold lysis and their membrane magnesium content. The most susceptible cells seem to have comparatively low levels of membrane-bound Mg^{2+} (156). As a result of these interesting observations, Möllby (124) speculated that hot-cold hemolysis occurs when glycerophospholipids in hydrolyzed membranes lose their capacity to maintain stabilizing ions at low temperatures. Thus, divalent cation stabilization is considered to be temperature dependent.

In summary, it appears that erythrocytes susceptible to lysis by beta-toxin have membranes that are nearly entirely composed of sphingomyelin at their outer periphery. Degradation of approximately 50% of this sphingomyelin causes instability of the membrane that ruptures after the cells are chilled. Although there is no precise explanation of low temperature-induced hemolysis, speculative suggestions by other investigators were discussed. All of these suggestions were within the framework of a general model for the action of phospholipases on erythrocytes.

Electron microscopic examination of beta-toxin-treated sheep and human erythrocyte ghosts revealed membrane shrinkage with the formation of numerous internal vesicles containing amorphous material (18). Bernheimer (17) suggested that the amorphous material might be the ceramide (*N*-acyl sphingosine) degradation product of sphingomyelin. The vesicles in the shrunken membranes were more extensive in the sheep ghosts than the human ghosts.

Similar ultrastructural changes of human and bovine membrane ghost preparations exposed to beta-toxin were observed by Low and co-workers (104) after applying the freeze-etching technique. Electron photomicrographs of treated ghost membrane preparations showed extensive invagination, the presence of vesicles, and raised particle-free areas in the hydrophobic fracture plane. Particle-free globules believed to consist of ceramide were also frequently seen within ghosts. Such aggregates were more frequent in bovine ghosts than in human ghosts. Similar aggregates were seen by Verkleij et al. (172) in freeze-etched sections of erythrocyte ghosts treated with sphingomyelinase C. This group suggested that the aggregates no longer functioned in maintaining the organizational structure of the outer phospholipid monolayer, thereby increasing fragility of the cell.

It was speculated by Low et al. (104) that the observed process of invagination with subsequent formation of vesicles would be expected to result from sphingomyelin hydrolysis if specific conditions were satisfied. These conditions included (i) sphingomyelin being preferentially located in the outer leaflet of the lipid bilayer and (ii) lateral migration of ceramide along the plane of the membrane into amorphous aggregates. If these conditional assumptions are valid, an inward collapse of the membrane would then occur to reduce lipid surface areas in the depleted outer leaflet of the membrane. The first conditional assumption of this hypothesis agrees with the finding that the degree of beta-toxin-induced hemolysis directly correlates with the amount of sphingomyelin in the outer membrane leaflet of susceptible cells. However, the second conditional assumption in the hypothesis by Low et al. (104) has been disrupted by Colley et al. (32). These latter workers claimed that ceramide, produced by the action of sphingomyelinase, remained at the site of hydrolysis and did not migrate to form amorphous aggregates. Their observation was based on their inability to detect so-called black dots by phase-contrast optics of ghost preparations treated with sphingomyelinase C. Alternatively, Bernheimer et al. (18) using phase-contrast microscopy did observe phase-dense droplets in beta-toxin-treated ghosts.

DELTA-TOXIN

Properties

Several groups of investigators beginning with Williams and Harper (186) studied a substance that became known as delta-toxin or delta-lysin. Production and purification of the toxin have been reviewed by Wiseman (190). The properties of the toxin were not defined accurately until investigators began working with pure toxin preparations. The problems encountered in working with impure preparations of delta-toxin were similar to those described above for alpha-toxin. The studies of Kreger et al. (95), Heatley (68) and Kantor et al. (78) revealed that delta-toxin had properties that were quite different from other membrane-damaging bacterial toxins with the exception of surfactin from *Bacillus subtilis*. Some of the unique characteristics of delta-toxin as pointed out by Bernheimer (17) include: (i) its relative hydrophobic nature, (ii) thermostability, (iii) a low degree of cellular specificity, (iv) inhibition by phospholipids, and (v) high degree of surface activity. Its wide spectrum of cytolytic activity includes lysis of bac-

terial protoplasts and spheroplasts, lysosomes, lipid spherules, mitochondria, and a wide number of erythrocytes (94, 95, 137).

Molecular weights ranging from 68,000 to 200,000 have been reported for delta-toxin. Kantor et al. (78) found that nonionic detergents disassociated the toxin into five subunits weighing approximately 21,000 each. Amino acid analyses from four different laboratories (68, 78, 95, 212) were in essential agreement and detected that histidine, arginine, proline, tyrosine, and cysteine were absent. The protein has an unusually high content of hydrophobic amino acids, and the relationship of this property to the toxin's mode of action will be discussed shortly.

Similar to other staphylococcal toxins, the protein is electrophoretically heterogeneous. The data concerning the electrophoretic migration pattern of delta-lysin are not in agreement (78, 95, 125, 190). It has been observed to fractionate into as many as four components after electrophoresis. However, the pI of the major component appears to be in the pH range of 9.0 to 9.6 (78, 95, 125). Kantor et al. (78) have suggested that the unusual electrophoretic behavior of delta-toxin is due to its unusually high degree of aggregation and charge masking.

There has also been controversy concerning the antigenicity of the toxin. Several investigators were unable to raise antibody against purified delta-toxin (60, 64, 78) in spite of the fact that serum can neutralize delta-toxin activity (39, 60, 109). However, data by Donahue (39) and Kapral (79) indicated that either serum lipoproteins or serum phospholipids, and not immunoglobulin, caused this neutralization. In extended studies, Fackrell and Wiseman (50) injected pure delta-toxin into rabbits. After removing the nonspecific inhibitors of delta-toxin from the serum of these rabbits, these workers were able to identify anti-delta-lysin antibody. The purified antibody showed a single precipitin line with purified antigen in Ouchterlony double-diffusion analyses. However, in spite of this work, the antigenicity of the toxin still remains in doubt (81).

Many of the properties described above indicate that delta-toxin disrupts biological membranes by means of detergent-like action. Kinetic analyses of the clearing of suspensions of leukocytes and platelets treated with delta-lysin failed to detect a lag period before changes in membrane permeability (95, 175). In addition, lysis of human erythrocytes by delta-toxin occurs rapidly, without an obvious lag period, and is temperature independent (81). Such action is typical of detergents. Similar evidence for the detergent-like activity of the toxin was reported by Thelestam et al. (170), who observed that leakage of [^{3}H]uridine from human diploid fibroblasts was detectable immediately after addition of the toxin and was independent of temperature. The reaction was almost identical to that seen after treatment of the fibroblasts with the nonionic detergent Triton X-100. Morphological changes in fibroblasts treated with Triton X-100, delta-toxin, and melittin, the surface-active lytic toxin from bee venom, were similar. The cytoplasm of the fibroblasts became highly granular and somewhat shrunken, but nuclei appeared normal (168). However, Triton X-100 differed from both melittin and delta-toxin in its biological action. Triton-treated cells showed an immediate rapid release of both labeled ribonucleic acid (molecular weight, 2×10^5) and a smaller-molecular-weight (1×10^3) labeled nucleotide. In contrast, melittin and delta-toxin displayed an initial preferential release of nucleotide label. After prolonged incubation, both labeled markers were released at similar rates. This data indicated that Triton X-100 produced larger initial membrane pores than either melittin or delta-toxin. However, with increased incubation time, melittin and delta-toxin induced lesions that approached the dimensions of those made by Triton X-100. Further evidence for the detergent-like properties of delta-lysin was demonstrated by Rahal (137), who showed that Triton X-100, deoxycholate, and delta-toxin all caused similar metabolic changes in mammalian mitochondria.

A study of the chemical architecture and physical properties of delta-toxin would be expected to reveal further insight about its detergent-like nature. Bernheimer (17) suggested that, if the high hydrophobic amino acid content of the toxin were confined to a single region, the molecule, like melittin, would be amphipathic and strongly surface active. Some similarities in the biological activities of melittin and of delta-toxin have been discussed above. A significant study of the surface properties of delta-toxin was recently made by Colacicco et al. (30). These investigators observed that delta-toxin behaved as both a phospholipid and a protein in its ability to form a film at air-water interfaces. The toxin resembled a phospholipid in forming an unusually stable, lipid-like, high-pressure film when spread from an organic solvent (chloroform–methanol, 2:1, vol/vol) onto distilled water. On the other hand, the toxin resembled a protein when its ability to spread as a film from water decreased with increasing pH. The films were stable over the entire pressure range. The unusually high surface potential of the toxin was similar to that of saturated lecithins. The high

surface potential was thought to be due, in part, to a dense packing of hydrophobic lipid-like areas of the toxin. Fluorescence studies with 1-aniline 8-naphthalene sulfonate indicated that the toxin was capable of binding to lipids. These observations led Colacicco and co-workers (30) to suggest that delta-toxin had the structural characteristics of a membranophilic protein with the ability to readily insert itself into hydrophobic membrane structures. The high content of hydrophobic amino acids and lack of disulfide bridges was thought to give the protein flexibility and conformational versatility that are probably required for the transport of delta-toxin across membranes. Observations by Kapral (81) indicated that fatty acids were involved with the binding of toxin to membrane. Palmitic acid and related fatty acids were believed to be optimal for binding. Cells exposed to palmitic acid and separated from excess lipid became more susceptible to delta-toxin.

Wiseman and Caird (192) suggested that the mode of action of delta-lysin involved phospholipase C activity. This was based on results showing that purified delta-toxin caused the liberation of organic phosphorus from erythrocytes in direct proportion to their hemolytic sensitivity. However, other workers were unable to demonstrate phospholipase C activity in their toxin-purified preparations (68, 79, 95, 137). Furthermore, the reaction kinetics of delta-lysin do not suggest an enzymatic mode of action (81, 112). In summary, the wide spectrum of cytolytic activity, chemical architecture, high surface activity, and reaction kinetics of delta-toxin provide strong evidence to indicate that the toxin lyses cells by way of a detergent-like mechanism of action. The membrane receptor site for delta-toxin is believed to be a straight-chain fatty acid with 13 to 19 carbons.

Interrelation in Disease

Kapral and co-workers (83) demonstrated that delta-toxin inhibited water absorption in rabbit and guinea pig ileum. This effect is blocked by lecithin, a known inhibitor of delta-toxin (133). The toxin also caused histological damage to guinea pig ileum at high doses and elevated cyclic adenosine 3',5'-monophosphate levels of the ileum at low doses (131). In these respects, delta-toxin behaved similarly to cholera toxin. However, extended studies suggested that stimulation of intestinal cyclic adenosine 3',5'-monophosphate levels by delta-toxin was mediated by a mechanism different from that induced by cholera toxin (132). In contrast to cholera toxin, delta-toxin caused significant changes in intestinal ion transport long before cyclic adenosine 3',5'-monophosphate levels were seen to increase. Although the mechanism responsible for the effects of delta-toxin on guinea pig ileum remains unknown, it was suggested that the toxin acted directly on the cell membrane, causing a primary effect on ion transport which in turn might affect intracellular cyclic adenosine 3',5'-monophosphate concentration. Since delta-toxin causes an immediate increase in unidirectional, intestinal ion fluxes and the increase in cyclic adenosine 3',5'-monophosphate levels occurs after an approximate 1-h lag period, it is likely that the elevation in the cellular cyclic adenosine 3',5'-monophosphate levels is a secondary response to the toxin (133). The interesting results of these studies indicate that delta-toxin might play a role in the pathogenesis of intestinal diseases ranging from mild diarrhea to severe enteritis.

GAMMA-TOXIN

Overview

Although gamma-toxin was described in 1938 by Smith and Price (155) and proposed to be a distinct entity by Marks in 1951 (110), it attracted little attention in subsequent years. There were some investigators who doubted and even denied its existence (42, 73). However, current investigations from four independent laboratories have firmly established the existence of gamma-toxin (52, 63, 126, 164). All of these studies were done with staphylococcal strain 5R, which produces gamma-toxin as its main cytolysin. The production and purification of gamma-toxin have been adequately reviewed by Wiseman (190).

The spectrum of activity of gamma-toxin against susceptible erythrocytes is different from that of the other staphylococcal membrane-damaging toxins. Gamma-toxin lyses rabbit, human, sheep, goat, dog, and fowl erythrocytes, but not those of the horse (52). There is general agreement that, among susceptible erythrocytes, rabbit erythrocytes are the most sensitive and those of fowl the least sensitive to toxin action (52, 126). The toxin is also capable of lysing human leukocytes and is cytotoxic to human lymphoblast cells (52).

Little is known about the cytotoxic effects of gamma-lysin. It was reported to be lethal for rabbits (155) and mice (63). However, a recent study by Fackrell and Wiseman (52) indicated that 100 μg of purified gamma-lysin had no effect after subcutaneous inoculation into either guinea pigs or rabbits. The same dose administered either intraperitoneally or intravenously also had no effect on mice. However, guinea pigs were instantaneously killed after intracardial ad-

ministration of 50 μg of toxin. Postmortem analyses indicated massive hemorrhage of the kidney and intestinal serosal surfaces, accompanied by lysis of erythrocytes in the major veins and arteries.

The antigencity of gamma-toxin has been adequately documented (52, 63, 165). Fackrell and Wiseman (52) showed that antibody against gamma-lysin interacts with the toxin to produce a single line of precipitation after diffusion on Ouchterlony agar. There was no evidence of cross-reaction between gamma-antitoxin and other staphylococcal cytolysins. Taylor and Plommet (165) have reported that the estimation of serum gamma-antilysin titers in humans can be useful in the diagnosis of staphylococcal osteomyelitis.

Properties

Guyonnet and Plommet (63) found that gamma-toxin separated into two components after purification by hydroxyapatite chromatography. The two components interacted synergistically to cause either hemolysis or toxicity in mice. These findings were confirmed by the work of Taylor and Bernheimer (164) who showed that gamma-lysin was composed of two separate proteins which they designated as component I and component II. Component I had a molecular weight of 29,000 and an approximate pI of 9.8. Component II had a molecular weight of 26,000 and an approximate pI of 9.9. Components I and II could be separated by hydroxyapatite chromatography and depended on one another for biological activity. The minute difference between the isoelectric points of components I and II explains why Möllby and Wadström (126) isolated the toxin as a single component after isoelectric focusing. In addition, the separation of these components by methods depending on molecular weight differences would not be expected since the molecular weights of components I and II are so similar. Therefore, it was no surprise that Fackrell and Wiseman (51) isolated the toxin as a single component after purification by differential ultrafiltration and fractionation on a Sephadex G-75 gel column. The single component had a molecular weight of 45,000 and an isoelectric point of 6.0 (52). Gamma-lysin had a sedimentation coefficient of 2.65, and amino acid analysis revealed that it had low levels of methionine and histidine (52). A striking feature of the amino acid analysis revealed that gamma-toxin resembled alpha- and delta-toxin because it had high levels of aspartate and lysine and no detectable cysteine. All of the protein's methionine was believed to be located at the N-terminal position. Gamma-toxin is inactivated after heating at 55°C for 10 min (71). The properties of gamma-toxin are summarized in Table 5.

Very little is known about the mode of action of gamma-toxin. The fact that two protein components are required for biological activity might suggest that the toxin has a distinct and complex mechanism of action compared to the other staphylococcal membrane-damaging toxins. A wide number of different agents have been observed to inhibit the toxin's action, but these observations have done little to explain the toxin's mode of action.

Jackson (71), but not Guyonnet and Plommet (63), found that the activity of gamma-lysin is inhibited by either ascorbic acid or cysteine. The toxin was also observed to be inactivated after interaction with sulfonated polymers such as agar, heparin, and dextran sulfate (164, 175). Taylor and Bernheimer (164) suggested that inactivation was caused by binding of the basic toxin to the acid groups of the sulfonated polymers.

Similar to streptolysin O and other sulfhydryl-activated hemolysins, gamma-lysin is inactivated by low levels of cholesterol (164). Inhibi-

TABLE 5. *Purification and properties of gamma-toxin isolated from staphylococcal strain 5R*

Main step(s) in purification procedure	No. of components isolated	Mol wt	pI	Reference
Ultrafiltration, gel filtration	1	45,000	6.0	Fackrell and Wiseman (51, 52)
Ion-exchange chromatography	2	—[a]	—	Guyonnet and Plommet (63)
Isoelectric focusing	1	—	9.5	Möllby and Wadström (126)
Ultrafiltration, ion-exchange chromatography	2	29,000 (component I) 26,000 (component II)	9.8 (component I) 9.9 (component II)	Taylor and Bernheimer (164)

[a] —, Not reported.

tory sterols for sulfhydryl-activated toxins must have a free β-hydroxyl group on carbon 3 of the pentanephenanthrene nucleus. However, gamma-toxin is inhibited by cholesterol acetate on which the 3-β-hydroxyl group is esterified. Therefore, gamma-toxin and the sulfhydryl-activated toxins are probably inhibited by cholesterol through different mechanisms. Similar to delta-toxin (79, 95), gamma-toxin is inhibited by phospholipids (52, 164). In addition, it was mentioned previously that there was a striking similarity between the amino acid compositions of these two toxins. Therefore, it is tempting to suggest that gamma-toxin might exert its membrane-damaging effects through high surface activity.

An enzymatic mode of action for gamma-toxin also has been considered. The toxin is inhibited by ethylenediaminetetraacetic acid and citrate, which indicates that its activity may be dependent upon a cation (52). Its reaction kinetics are compatible with those of an enzymatic reaction, and its hemolytic activity occurs at an optimum pH of 7.0 and an optimum temperature between 27 and 45°C (52). Fackrell and Wiseman (52) observed that human erythrocyte membranes treated with gamma-toxin released acid-soluble phosphorous and nitrogen at a linear rate. However, both Fackrell and Wiseman (52) and Taylor and Bernheimer (164) observed that the toxin did not affect the major lipids extracted from human erythrocyte membranes. In addition, membrane protein did not inhibit gamma-toxin-induced hemolysis nor did the toxin degrade azocoll or tosyl arginine methylester (52). Therefore, it appears that the toxin lacks both lipase and proteolytic activity.

No morphological data are available to assess the membrane-damaging effect of the toxin. However, the kinetics of cytoplasmic leakage of human fibroblasts treated with gamma-toxin were similar to those observed for alpha-toxin and beta-toxin (169). This would indicate that the size of the membrane lesions inflicted by gamma-toxin is similar to that caused by alpha- and beta-toxins. In summary, much more meaningful data will be needed before a plausible mechanism of action can be proposed for gamma-toxin. At this time, it appears that there is more evidence to argue against rather than for an enzymatic mode of action.

LEUCOCIDIN

Overview

In 1932, Panton and Valentine (134) described an extracellular staphylococcal product that exerted an effect on leukocytes, but not on erythrocytes. In honor of its discoverers, this toxin was named Panton-Valentine leucocidin by Wright (210). Its exclusive and selective action on polymorphonuclear leukocytes and macrophages of rabbits and humans became evident in subsequent years. No other cell types appear to be susceptible. Woodin (198, 199) found that highly purified leucocidin disassociated into two components after migration on a carboxymethylcellulose column. These components, designated as F (fast) and S (slow), are biologically inactive alone, but interact synergistically to induce leukocytosis.

The morphological changes that were inflicted by leucocidin on rabbit leukocytes were described by Gladstone and Van Heyningen (59). Light microscopy revealed that, after 60 s of treatment, cytoplasmic streaming reverted to Brownian motion. Two minutes later, loss of pseudopodia, rapid degranulation, and cellular swelling were observed. At a later time, the nuclei ruptured and the cell gave the appearance of an empty vacuole. Woodin (202) reported that these morphological alterations were dependent upon calcium, since electron photomicrographs of cells treated with toxin in the absence of calcium showed no gross morphological change. Ethylenediaminetetraacetic acid protected the cell against toxin-induced damage.

In vivo, the sole effect of leucocidin is on the leukocytes of humans and rabbits (74). High doses of toxin administered to rabbits for several days significantly stimulated the granulocyte system (75). A single injection of leucocidin caused pronounced granulocytopenia which was followed by extensive granulocytosis and increased serum lysozyme levels (75). One to 2 days after leucocidin injection, there was an increased proliferation of granulocyte precursors (162). However, Jeljaszewicz (74) has pointed out that proliferation of granulocyte precursor cells could have either resulted from direct toxin action on these cells or from a secondary effect due to damage of mature cells. In a recent study, Szmigielski and Jeljaszewicz (160) observed that rabbits receiving cytostatic agents suffered from marked granulocyte system impairment which was manifested by decreased granulocytes in the peripheral blood and by a decrease in the bone marrow granulocyte reserve. However, administration of leucocidin to these animals during the period of myelosuppression resulted in partial protection against the effects of the cytostatic agents and stimulated regeneration of the granulocyte system.

Interrelation in Disease

It has been established that leucocidin caused marked leukocytosis, but the relationship of this

event to human staphylococcal disease has not been determined. Gladstone et al. (58) have suggested that antibodies against leucocidin are essential for resistance against staphylococcal infections. This was supported by their finding elevated levels of leucocidin antitoxin in patients with staphylococcal diseases. Staphylococcal production of leucocidin is greatly enhanced in vivo (57). It has been suggested by Rogers and Tompsett (143) that leucocidin enhances staphylococcal invasiveness by allowing the organism to resist phagocytosis.

Properties

Leucocidin is optimally produced by strain V8 under the conditions described by Woodin (197). The cells were grown in a medium containing amino acids, salts, lactate, glycerophosphate, and a diffusate of yeast extract. Highly purified Panton-Valentine leucocidin consisting of the individual F and S components was first obtained by Woodin (197, 198). The toxin was initially precipitated with $(NH_4)_2SO_4$ and further purified by fractionation with $(NH_4)_2SO_4$ and by chromatography with hydroxyapatite. The F and S components were then separated after two-stage chromatography with carboxymethylcellulose.

The F component crystallizes at pH 7 in phosphate buffer (202). The S component is soluble at pH 7, but can be obtained in a crystalline state in solutions containing a high concentration of $(NH_4)_2SO_4$. The molecular weights for the F and S components were determined to be 32,000 and 38,000, respectively, after analysis on the analytical ultracentrifuge (202). Both proteins sedimented with single symmetrical peaks after ultracentrifugation, but the boundary spreading indicated that both components were polydisperse. This was presumably due to the presence of various conformational forms for each component. Both proteins had sedimentation coefficients of 3S. After electrophoresis, the S component was found to have the higher charge. Wadström et al. (176) reported that leucocidin had an isoelectric point of 9.0. The protein is thermolabile and loses nearly all activity after incubation at 60°C for 10 min (59). Approximately 50% of the toxin's activity is lost after standing either at room temperature or at 4°C for 3 days (75). Leucodicin preparations can be stored at −20°C or lyophilized for several months without any loss of activity (75).

At low ionic strength, both F and S proteins undergo partial polymerization (205). After polymerization, approximately 30% of each component was sedimentable after centrifugation. The sedimented material dissolved in solutions of physiological ionic strength. Woodin (202) interpreted this to mean that each component existed in different conformational forms in solutions of physiological ionic strength; but when the ionic strength is lowered, some of these conformational forms polymerize.

Nearly all that is known about the extremely complex mode of action of leucocidin has been derived from the work of Woodin and co-workers (199–209). The primary action of the toxin is exerted on the cell membrane. Three general approaches have been used to study the mode of action of leucodicin. These include: (i) studies of toxin-induced permeability changes, (ii) studies on leucocidin-leukocyte membrane interaction, and (iii) studies on the interaction between the toxin and free phospholipids. Following is a discussion of these areas of study.

It appears that the only unique response of leukocytes to leucocidin is an altered permeability to cations. Treated cells have an immediate loss of K^+ ions, but there is no loss of larger molecules such as phosphates or nucleotides (202). When calcium is present, it is accumulated in the cell, and magnesium is displaced. If chelating agents are present in the medium, calcium is lost from the cell. The permeability changes were accompanied by cellular accumulation of orthophosphate at the expense of adenosine 5′-triphosphate. There was also a decrease in the rate of nucleotide turnover. The changes inflicted by leucocidin were believed to be due to a structural change in the membrane which leads to increased permeability. The possibility of leucocidin-induced damage occurring from inhibition of active transport was ruled out. Woodin and Wieneke (207) demonstrated that the activity of leucocidin was enhanced in the presence of diisopropyl phosphofluoridate. Evidence was provided to show that diisopropyl phosphofluoridate interfered with the potassium pump, but not in the same manner as leucocidin. Diisopropyl phosphofluoridate was believed to function by preventing the cell from reversing the membrane damage inflicted by leucocidin.

In other studies, Woodin and Wieneke (204) studied the interaction between isolated leukocyte membranes and leucocidin. At physiological ionic strength, the toxin was inactivated in the presence of the free membrane. A small amount of binding was observed, mainly involving the S component. Most of the inactivated leucocidin was detected in solution. At low ionic strength, interaction between toxin and membrane led to polymerization of leucocidin. Approximately 70% of the added leucocidin polymerized, and polymerization did not increase with higher

membrane concentration. Both inactivation and polymerization were dependent upon synergistic interaction between the two components. During the polymerization reaction, the membrane appeared to function as a catalyst but was not altered by the reaction. Alternatively, the inactivation reaction was believed to cause a membrane change. Woodin and Wieneke (204) concluded from their observations that the inactivation reaction was a more significant event than the polymerization reaction in the mode of action of leucocidin.

Extended studies indicated that lipids were involved in the inactivation and polymerization of leucocidin. At low ionic strength, phosphatidylserine, diphosphoinositide, triphosphoinostide, and phosphatidylcholine were all able to polymerize the F protein (206). Polymerization is reversible at higher ionic strength. These results suggested that the F component interacted with charged glycerophospholipids containing two esterified fatty acids. The reaction was dependent upon conditions that induced electrostatic repulsion between the polar hydrophilic regions of the cell membrane. In extended studies (202) it was demonstrated that the F component was polymerized by diglyceride which was prepared by enzymatic hydrolysis of phosphatidylserine. This indicated that the leucocidin component interacted with the hydrophobic region of a fatty acid side chain of the phospholipid. Alternatively, the S component is not polymerized by interacting with the hydrophobic region of phospholipids. Instead, the S component becomes polymerized after interaction with the F component.

Triphosphoinositide was the only free phospholipid observed to inactivate leucocidin. The S component was inactivated at physiological ionic strength. The F component was inactivated at low ionic strength and remained inactive after the ionic strength of the reaction mixture was raised to physiological values. Leucocidin induced a conformational change of triphosphoinositide when lipid was either in the free state or present in the leukocyte membrane. The leukocyte cell membrane contains enough triphosphoinositide to account for the inactivation of leucocidin (207). However, nonsusceptible erythrocytes also contain adequate amounts of triphosphoinositide needed to inactivate the toxin. This indicates that inactivation is dependent upon accessibility of the phospholipid to the toxin. It was estimated that the outer leaflet of the leukocyte membrane contained approximately 1% triphosphoinositide. In light of the above evidence, triphosphoinositide would appear to be the likely target site for leucocidin.

The above discussion concerning the polymerization and inactivation of leucocidin by membranes and free phospholipids under conditions of differing ionic strength indicate the toxin's mode of action is extremely complex. Woodin (201) used his experimental data to propose the following mode of action for the toxin. Initially, the F component migrates into a region of the membrane with a low dielectric constant and undergoes a conformational change into an expanded form. This is followed by interaction between the fatty acid side chains of phospholipid and a hydrophobic region of the F component. The S component is then adsorbed onto the altered surface of the F protein, allowing for interaction between the S protein and triphosphoinositide. These reactions lead to the formation of a membrane pore accompanied by a loss of the region of low dielectric constant and a return of the F component to its original "compact" form. Inactivated F protein is then released from the fatty acid chains of triphosphoinositide accompanied by desorption of the inactivated S component.

In extended studies designed to further elucidate the mechanism of action of leucocidin, Woodin and Weineke (203, 208, 209) examined the effect of the toxin on membrane-associated enzymes. It was found that acylphosphatase activity was stimulated by leucocidin. Since the enzyme was K^+ sensitive, it was believed to be a component of the potassium pump. Since triphosphoinositide was the only acidic phospholipid observed to inhibit acylphosphatase activity, it was suggested that leucocidin could upset membrane permeability by altering the relationship between potassium-sensitive acylphosphatase and membrane phospholipids.

The possible role of cyclic adenosine 3',5'-monophosphate in the mode of action of leucocidin was also investigated (203). In rabbit leukocytes, most of the adenylate cyclase activity was found to be associated with the plasma membrane. Toxin-membrane interaction resulted in the stimulation of adenylate cyclase activity. Woodin (203) postulated that there was a close association between acylphosphatase and adenylate cyclase and that their stimulation by leucocidin results from production of an ion channel following specific perturbation of membrane phospholipids.

In summary, Woodin and Wieneke have uncovered a vast amount of information pertaining to the mode of action of leucocidin. It was possible to formulate significant aspects of these studies into a model that explained how two toxin components interacted syngeristically to alter membrane permeability. Neither the F nor

the S component appeared to have any direct enzymatic activity during interaction with the leukocyte membrane.

SUMMARY AND CONCLUSIONS

Although there are still many unanswered questions about staphylococcal toxins, it is hoped that this review has been successful in conveying the significance and considerable progress of the recent work with nonenteric toxins of *S. aureus*. Much of the progress made in the research with these toxins can be attributed to the awareness of the necessity to work with pure toxin preparations. The analysis of a toxin's behavior can be misinterpreted as a result of even trace contamination of a purified toxin preparation (113). One of the most challenging areas of bacterial toxinology is defining a toxin's mode of action. The ultimate description of a toxin's mode of action must be in molecular terms that describe biochemical interactions. However, important clinical and ultrastructural observations about a toxin's biological behavior can provide important clues about a toxin's molecular behavior.

Although many secrets about the mode of action of staphylococcal toxins still need to be uncovered, it appears that each toxin has a distinct mechanism for causing molecular alterations. Beta-toxin enzymatically degrades membrane sphingomyelin. Delta-toxin affects membranes by way of its detergent-like properties. Leucocidin works through the synergistic action of its F and S components. These components interact with leukocyte membrane substances, and this interaction induces an altered permeability to cations. ET, either directly or indirectly, disrupts the factors which are responsible for the adhesion of cells within the stratum granulosum of neonatal mice and newborn infants. Any further comments about the mechanism of action of ET at this time would constitute unwarranted speculation. The isolated bits of information that are available about alpha-toxin cannot be assembled into precise terms that would define the mode of action of this toxin. A known membrane receptor for alpha-toxin has not been positively identified. The molecular events which lead to membrane disruption as a result of alpha-toxin–membrane interaction have also not been positively identified. There is a controversy about whether these events involve proteolytic activity or detergent-like activity.

Several experimental approaches have been important in yielding valuable information about toxin action. Ultrastructural studies have provided visual accounts of the biological sequence of events that are induced by the membrane-damaging toxins and ET. Permeability changes and the size of the lesions that are induced by membrane-damaging toxins have been detected by tests that measure the leakage of intracellular ions and low-molecular-weight compounds. Experiments designed to measure changes in the activity of membrane-bound enzymes have provided vital clues about the action of leucocidin. Analyses of changes in the phospholipid content of toxin-treated membranes have explained much about the action of beta-toxin. Analyses of the properties of delta-toxin and alpha-toxin indicated that these toxins had detergent-like action. Observations relating to the binding kinetics and polymerization of toxin protein have provided valuable information about the molecular action of alpha-toxin and leucocidin. Artificial phospholipid model membrane systems have been a great aid in studying the surface activity of alpha-toxin and delta-toxin.

Aside from deciphering toxin mode of action, another challenging and exciting problem of bacterial toxinology is searching for a relationship between toxins and disease. Since *S. aureus* produces a multitude of different toxins and caused disease in nearly every organ of the body, it serves as an ideal model system to study this relationship. It is possible to demonstrate experimentally that inoculation of a purified preparation of staphylococcal ET into the skin of a neonatal mouse will induce all the manifestations of the SSSS. However, it is now realized that, in nearly all circumstances, pathogenicity is multifactorial and is rarely caused by a single determinant. For instance, before the onset of the SSSS, the etiological agent must penetrate body surfaces, colonize, and produce an infection. Susceptibility of the host is another important factor in the establishment of this disease. The role of the membrane-damaging toxins in disease is not as clear as it is for exfoliative toxin. The membrane-damaging toxins most likely play an important role in the initial stage of infection which involves multiplication and dissemination of *S. aureus* as a result of localized tissue damage. For example, it has been suggested that leucocidin enhances staphylococcal invasiveness by allowing the organism to resist phagocytosis (143).

The development of adequate experimental animal model systems to study toxin-disease relationships will be an important challenge to future investigators. Such model systems will aid in determining the role of staphylococcal toxins in the impairment of tissues and host defense systems. Thus, the advantage of an in

vivo system over an in vitro system becomes obvious. Nearly everything that is known about the relationship between ET and the SSSS was derived from studies with the experimental mouse model. However, a thorough and complete description of a toxin's role in pathogenicity must take into account significant observations from both in vivo and in vitro studies. In conclusion, recent progress from studies with nonenteric staphylococcal toxins has done much to enhance our understanding of bacterial toxinology. It seems certain that continued studies with nonenteric staphylococcal toxins will compose a highly significant aspect of the future research efforts in microbiology.

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