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Virulent and Avirulent Encapsulated Variants of *Staphylococcus aureus*

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There are at least two serologically distinct capsular types of coagulase-positive *Staphylococcus aureus*. Until now, unequivocal evidence for encapsulation of the Smith diffuse variant was lacking. However, the data presented in this paper provide definitive details of encapsulation of the Smith strain. A marked difference in LD_{50} values for the two serologically distinct capsular types of *S. aureus* was demonstrated. The paradoxical behavior of these two strains suggested that the host was resistant to one and was susceptible to the other. A survey of the carriage incidence in mice for staphylococci and staphylococcal capsular antibodies disclosed the presence of staphylococci and capsular antibodies in these animals. The capsular antibodies detected were reactive against only one of the capsular types of *S. aureus*. None of the sera from the mice surveyed possessed capsular antibodies against the Smith diffuse variant, but the average incidence for the capsular antibodies against the wound mucoid type was 46%. We postulated that the susceptibility of the mice to the Smith diffuse variant was caused by the absence of protective, type-specific capsular antibodies. Conversely, the resistance of the mice to the wound mucoid staphylococci may have been a result of the presence of type-specific capsular antibodies.

The results given in this paper are concerned with the paradoxical behavior of two encapsulated strains of *Staphylococcus aureus*, one that is virulent for laboratory mice and one that is not. The virulent strain, the Smith diffuse variant, is commonly accepted as encapsulated, but some investigators have failed to elicit a specific capsular reaction with this strain (6, 11-12). The avirulent strain, the wound strain isolated by Wiley (18), exhibited a specific capsular reaction. Although the wound strain of Wiley was less virulent for mice than the diffuse variant of Smith, it gave a positive specific capsular reaction, which, in our opinion, is the most definitive method for demonstrating capsules of *S. aureus*. We undertook these studies in an attempt to explain the possible paradox involved when one considers that both strains of *S. aureus* are encapsulated, but only the Smith diffuse variant was virulent for mice.

MATERIALS AND METHODS

Strains. The strains used were wound mucoid strain described by Wiley (18), and a variant of that strain obtained by testing colonies and selecting the one that was butyrous rather than mucoid. The wound variant did not exhibit a specific capsular reaction. M. G. Koenig and S. I. Morse supplied the compact and diffuse Smith strains that we used. All the strains were grown in serum soft agar to observe their colonial morphology (3, 4).

Media and cultivation. Buffered nutrient glycerol broth (BNG broth), buffered nutrient glycerol agar (BNG agar), and the semisynthetic medium previously described by Wiley (18) were employed. The semisynthetic medium was slightly modified for the growth of the Smith strain. Dextrose (1%) was substituted for the glycerol. A starter flask of 50 ml of semisynthetic medium was inoculated with a loopful of staphylococci that had previously been cloned and grown on BNG agar for 18 to 24 hr. The flask was incubated at 37 C on a reciprocating shaker (Eberbach & Son Co., Ann Arbor, Mich.). When growth had begun in the starter flask, the contents were aseptically transferred into 1 liter of semisynthetic medium. The *p*H was maintained at 7.0 to 7.4 by addition of sterile 1 N KOH several times daily. After cessation of growth, a Gram stain was made. Formalin was then added to a final concentration of 0.5%, and the cultures were once again returned to the shaker for 24 hr. Each culture was tested for sterility, by plating 1 ml onto a sheep blood-agar plate.

Mannitol Salt Agar (Difco) was used for primary isolation of *S. aureus* from rectal and throat swabs. Single colonies were selected from the Mannitol Salt Agar and streaked onto BNG agar plates. The colonies were Gram stained and tested for coagulase production.

Specific capsular reaction. The specific capsular re-

action was conducted by placing one loopful of antiserum on a vaseline-rimmed cover slip and mixing a loopful of BNG broth culture (6 to 8 hr) of staphylococci with it. A loopful of aqueous methylene blue was also added. The second procedure utilized an 18-hr BNG agar culture of staphylococci; a small portion of a single colony was picked and mixed with the antiserum, and methylene blue was added. All specific capsular reactions were read at 1, 2, and 24 hr.

Coagulase tests. Tube coagulase tests were run as previously described (18). For quantitative determination of free coagulase, the method of Yotis and Ekstedt (20) was followed; human plasma diluted 1:3 with saline was used in all coagulase tests.

Quantitation of α-hemolysin. To quantitate the amount of α-hemolysin in the broth supernatant fluids, the procedure of McFarlan (8) modified by Jackson (5) was used. The organisms were grown at 37 C for 48 hr, in an atmosphere of 30% carbon dioxide. Fresh rabbit cells (2%) were used in the titrations.

Preparation of mucin. Granular mucin type 1701-W, lot no. 123810 (Wilson Laboratories, Chicago, Ill.), was prepared by the method used by Miller (9). The mucin was blended in a Waring Blendor. A 5% suspension was employed for virulence enhancement.

Preparation of vaccines and injection of animals. The staphylococci were harvested from the semisynthetic broth by centrifugation at 4,080 × *g*, using a Servall centrifuge (model SS-4) and a GSA rotor. The pellet was washed three times in buffered saline (0.85%, w/v), and was finally suspended in the same solution. This constituted the stock vaccine. The suspensions were adjusted, as needed, to an optical density difference of 0.67, as compared with a water blank, at 515 mμ in a Bausch and Lomb Spectronic-20 colorimeter. The suspension was then diluted 1:20.

Equal volumes of Freund's incomplete adjuvant and diluted cells were mixed and sonically vibrated with a model S-77 Sonifier with a 90-w output (Branson Sonic Power, Danbury, Conn.) until a thick white emulsion was obtained. A portion (0.5 ml) of the suspension was injected into each hind footpad of a 2- to 3-kg albino rabbit. After 1 week, additional injections were given subcutaneously at weekly intervals, doubling the concentration of antigen each time. The animals received from 8 to 12 injections. A similar procedure was used for the immunization of New Hampshire red roosters—antigens were injected in the breast muscle.

Preparation of suspensions. Much of the difficulty encountered in determining accurate plate counts of encapsulated staphylococci lies in adherent properties caused by their mucosity. In this study, sonic treatment of the suspensions was used to break up the clumps of bacteria. A Branson Sonifier, equipped with a ⅛-inch (0.3-cm) micro tip stephorn, was used. We found that suspensions sonically treated for 2 min, with the power input at a setting of 2 and an amperage reading of 3.5 to 4.0 amps, gave the highest viable counts.

Determination of LD_{50}. Adult male Swiss albino mice weighing approximately 25 to 30 g were used in all experiments. The staphylococci were grown on BNG agar plates for a period of 18 to 20 hr, scraped from the plates, and diluted with BNG broth to an optical density difference of 0.33 to 0.41, as compared with a BNG broth blank at 515 mμ in a Bausch and Lomb Spectronic-20 colorimeter. These staphylococci were sonically vibrated as previously indicated and kept chilled in ice. They were then diluted in BNG broth dilution blanks with glass beads and mixed on a Vari Whirl Mixer (Van Waters & Rogers, Inc., Salt Lake City, Utah). The mice were injected by the intraperitoneal route with 0.50 ml of mucin (5%, w/)v, followed immediately by 0.25 ml of the appropriate dilution of *S. aureus*. We observed the mice daily for 14 days. The method of Miller and Taintor (10) was used to estimate the LD_{50} and the standard deviation.

RESULTS

Although other investigators (6, 7, 11–14) failed to show a specific capsular reaction with the Smith diffuse variant, recently we succeeded in producing the phenomenon with antisera made in roosters and rabbits.

Figure 1a illustrates a typical specific capsular reaction of the Smith diffuse strain after 18 hr of growth on a BNG agar plate. Homologous antiserum was prepared in roosters. Figure 1b depicts a negative reaction obtained with heterologous antiserum. The optimal conditions for demonstration of a positive specific capsular reaction differ for the wound strain and the Smith strain. The Smith strain shows capsules optimally from an 18-hr plate culture, and the wound strain shows capsules optimally in a 6- to 8-hr broth culture.

Table 1 reveals that each of the encapsulated strains has a serologically distinct capsule. The compact strain referred to in this paper is a nonencapsulated variant of the Smith strain.

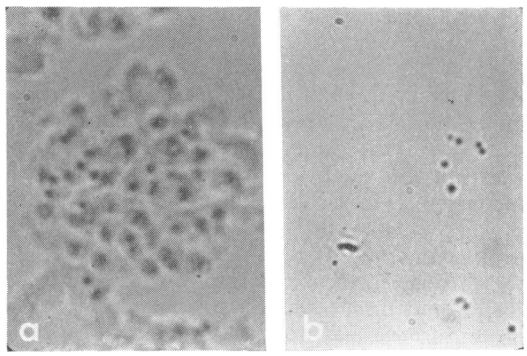

FIG. 1. *Specific capsular reactions of staphylococci:* (a) *positive specific capsular reaction of the Smith diffuse variant;* (b) *negative reaction obtained by using heterologous antiserum and Smith diffuse variant.*

TABLE 1. *Differentiation of two capsular types by the specific capsular reaction*

Strain	Antiserum prepared against	
	Encapsulated wound	Smith diffuse
Wound mucoid	+	−
Wound variant	−	−
Smith diffuse	−	+
Smith compact	−	−

The susceptibility of mice to the two encapsulated staphylococci and the lethality of mucin for mice were studied. Experiments in this laboratory indicated that high concentrations of mucin alone were lethal for mice. The effect of injecting 6, 5, 4, and 3% (w/v) mucin as an enhancer of staphylococcal virulence was investigated. The injection of 6%, but not 5% (w/v), mucin killed a few mice. We used the latter concentration. To obtain an accurate viable count of the number of staphylococci injected, sonic treatment of staphylococcal suspensions was employed. Suspensions sonically treated for 2 min yielded maximal plate counts; therefore, this was the standard treatment for all plate counts. In Table 2, LD_{50} values and their 95% confidence limits are expressed as the number of organisms producing a 50% kill. From these LD_{50} estimations given in Table 2, it is clear that a significant difference exists in the virulence of these two encapsulated strains for mice.

The virulent form of the Smith strain grows as a diffuse variant in serum soft agar. We believe that organisms growing in this fashion do so because the serum lacks antibody to the staphylococci (3, 4). The absence of antibody to a surface antigen of the Smith strain could explain the lowered resistance in mice to the Smith diffuse strain, and, conversely, the presence of antibody to such a surface component of the staphylococcus could account for the increased resistance in the host. The capsular antigen could be just such a surface component. If animals carried encapsulated staphylococci in their throat or rectum, then they could respond to the antigens of the microbe by producing antibodies. To explore this line of reasoning, we first assessed the carriage incidence of staphylococci in mice. Rectal and throat swabs were obtained from a group of mice and were then streaked on Mannitol Salt Agar plates. All mannitol-fermenting colonies were seeded to BNG broth for coagulase testing. Only those isolates that were coagulase-positive were considered to be *S. aureus*. The results of this survey are given in Table 3. The survey exhibited an incidence of staphylococcal

TABLE 2. *Estimations of LD_{50} in 25- to 30-g mice for the wound mucoid and Smith diffuse staphylococci*

Strain	No. of organisms	
	LD_{50}[a]	Confidence limits[a] (95%)
Smith diffuse	12	4–32
Wound mucoid	550,000	350,000–850,000

[a] The LD_{50} values and the 95% confidence limits are expressed as the number of organisms producing a 50% kill.

TABLE 3. *Carriage of Staphylococcus aureus in 60 mice*

Site of carriage	Coagulase-positive *S. aureus* carried[a] by	
	No. of animals	Per cent
Throat	19	31.6
Rectum	10	16.6
Throat and rectum	8 (16)[b]	13.3
Total	37	61.5

[a] Total number of strains isolated was 45.
[b] Sixteen isolates from a total of eight animals.

carriage of 61.5%. Of 60 mice, 37 harbored coagulase-positive staphylococci. A total of 45 coagulase-positive strains were isolated (some mice carried staphylococci in both the throat and rectum). Also, we noted that two of the staphylococcal strains isolated from the mice were naturally encapsulated, and these strains reacted with an antiserum prepared against the wound strain.

In view of these observations, a survey of the incidence of capsular antibodies in Swiss albino mice was undertaken. From the same group of mice used above, blood samples were obtained and the serum was tested for the presence of anticapsular antibodies against the wound mucoid strain and against the strain of *S. aureus* carried by the mouse from which the blood was removed. The results of these tests are presented in Table 4. Of the 60 sera tested, 33 gave a positive specific capsular reaction with the wound strain. This number represented 55% of the mice. None of the sera demonstrated a positive specific capsular reaction when tested against the *S. aureus* obtained from the same mouse as the serum. This finding, while unexpected, may be understood on the basis that the strains carried by the mice might not have been encapsulated and, hence, could not undergo a specific capsular reaction.

TABLE 4. *Incidence of specific capsular reactions in mouse sera*

Test strain	No. positive	Per cent
Wound mucoid	33/60	55
Autologous *S. aureus*	0/60	0

We conducted another survey on a group of 40 mice to ascertain whether antibodies against the encapsulated Smith strain and the encapsulated wound strain could be present simultaneously. The results of these specific capsular reactions are given in Table 5; 32.5% of the mice possessed capsular antibodies reactive with the wound strain, but none of the mice possessed capsular antibodies reactive with the Smith diffuse strain. The latter observation was not caused by the inability of the mice to make capsular antibodies against the Smith diffuse strain. All mice that survived the virulence tests of the Smith diffuse strain were injected with living Smith diffuse organisms. The first injection consisted of 500 staphylococci suspended in BMG broth. After 1 week, the mice were injected with 50,000 Smith diffuse organisms. The final injection was 2 weeks after the first, and a dose of 500,000 organisms was given to each mouse. The mice were bled 7 days later, and their sera were pooled. The pool from these mice exhibited positive specific capsular reactions with the Smith diffuse strain.

DISCUSSION

Tomcsik (17) believed that the term "quellung" or capsular swelling was a misnomer. His observations suggested that the majority of encapsulated organisms showed no detectable swelling of the capsule. Therefore, he proposed the term specific capsular reaction. Recently Baker et al. (1), using the electron microscope, noticed that the capsular reaction phenomenon, at least in the case of *Diplococcus pneumoniae* type 1, was a true swelling of the capsule. When specific antiserum and organisms were mixed, the antigen-antibody reaction occurred only at the surface of the capsule. However, the cell wall, cytoplasmic matrix, and nuclear material were not affected. Price and Kneeland (15, 16) elicited a specific capsular reaction with a strain of *S. aureus* (RLM), and called the reaction a capsular swelling; Wiley (18, 19) substantiated their findings. In prior papers, the term specific capsular reaction has been used instead of capsular swelling reaction or "quellung" because no data were available to indicate that an increase in capsular size occurred when specific antiserum and encapsulated staphylococci were combined.

TABLE 5. *Incidence of specific capsular reactions in mouse sera*[a]

Test strain	No. positive	Per cent
Smith diffuse	0/40	0
Wound mucoid	13/40	32.5

[a] Another survey for anticapsular antibodies in 40 additional mouse sera.

We have used the term specific capsular reaction to preserve uniformity with terminology used in previous papers.

Morse (12), Koenig (6), and Mudd et al. (13, 14) were unable to elicit a specific capsular reaction using the Smith strain, but we succeeded in this endeavor by employing both rooster and rabbit immune serum. Morse used the Smith diffuse strain in all of his experiments (*personal communication*). India ink preparations of this strain illustrated that the organism was surrounded by an envelope structure (12). Mudd et al. (13, 14) reported that the encapsulated Smith strain grew as a compact colony in normal rabbit serum. This staphylococcus was also surrounded by an envelope structure, as demonstrated by the India ink method. Although we do not dispute the validity of these observations, these data tend to obscure the basic question, namely, whether or not the virulent form of the Smith strain is encapsulated and grows as a diffuse colony in serum or plasma soft agar. Growth of the Smith strain in normal rabbit serum soft agar as a compact colony has usually been interpreted (6–7) as a characteristic of the nonencapsulated avirulent form of the Smith strain; but Mudd et al. reported that their culture of the Smith strain grew as a compact colony in normal rabbit serum soft agar. Electron photomicrographs of the Smith diffuse variant indicated the presence of a capsule (7). However, Koenig (6) was unable to find any difference in the diffuse or compact Smith strains when these strains were examined in India ink preparations. To use wet India ink preparations as the only criterion for the demonstration of encapsulated *S. aureus* could lead to confusion. It should be re-emphasized that the most definitive method for the demonstration of capsules of *S. aureus* is the specific capsular reaction.

Mudd et al. (13, 14) maintained that the encapsulated Smith strain used in their research should be considered as the prototype of encapsulated *S. aureus* strains and that the wound strain isolated in our laboratory should be considered as a representative of a phenomenon which they called the extracellular peripheral precipitation reaction (EPPR). Mudd et al. (13, 14) would

exclude the wound strain of *S. aureus* as being truly encapsulated because they would restrict true encapsulation to virulent naturally occurring strains. The wound strain in its encapsulated state was obtained by the method employed by Bigger, Boland, and O'Mera (2). Its virulence is indisputable (18). We have isolated naturally occurring encapsulated *S. aureus* of the wound type from rabbits, roosters, mice, and humans. Each isolate exhibited a positive specific capsular reaction upon initial isolation (18, 19). We devised and employed a virulence test in embryonated hens' eggs in our laboratory (18), which is well suited to the testing of the virulence of *S. aureus* isolates. In this test, the wound strain behaved as a virulent strain with an LD_{50} of less than 500 organisms. Our finding of anticapsular antibodies that react with the wound strain in normal rabbits, roosters, mice, and humans (18, 19) further supports the validity of our position. Apparently, the wound strain is a major capsular type of *S. aureus*, but not the only one. Evidence presented in this paper indicates, for the first time, that the Smith diffuse variant is also encapsulated, as shown by the specific capsular reaction. The wound strain and the RLM strain of Price and Kneeland represent a major capsular type against which antibodies are widely distributed (18, 19).

No detectable differences were found between the wound strain and the Smith diffuse variant, regarding the amount of hemolysin present in cultures. Differences in the amount of coagulase formed by each strain were not great enough to explain the difference in virulence observed. Apparently, the best explanation is that the mice were resistant to the wound strain because they carried it, and they had elaborated antibodies against it. Our tests did not reveal that mice carried the Smith capsular type of *S. aureus*, nor could we detect anticapsular antibodies against the latter.

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