Antiphagocytic Effect of the Capsule of *Staphylococcus simulans*

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An encapsulated strain of *Staphylococcus simulans* was observed to be more resistant to phagocytosis by human granulocytes than was a nonencapsulated strain. Phagocytosis of the encapsulated strain was enhanced by antiserum to *S. simulans*, but opsonic activity of antiserum was removed by absorption with *S. simulans* capsular material. The encapsulated strain of *S. simulans* was also more invasive than the nonencapsulated *S. simulans* in vivo. More encapsulated than nonencapsulated *S. simulans* were found in heart blood when equal numbers of organisms were injected intraperitoneally into mice. Invasion of the bloodstream of mice by encapsulated *S. simulans* was prevented by passive immunization (rabbit antiserum). Thus, the capsule of *S. simulans* inhibited phagocytosis in vitro and contributed to virulence in vivo.

Coagulase-negative staphylococci (CNS) are ubiquitous inhabitants of human skin and mucous membranes. The novobiocin-resistant *Staphylococcus saprophyticus* group can cause urogenital infections in sexually active adults (20, 33). The novobiocin-susceptible species of the *Staphylococcus epidermidis* group and *Staphylococcus simulans* are recognized more and more as important opportunistic pathogens in immunocompromised patients, e.g., premature neonates and leukopenic cancer patients, and in hospitalized patients (8, 9, 11, 28). The strains involved are especially characterized by their abilities to adhere to polymer surfaces and to produce a slime substance (8, 9, 24, 25).

It is further evident that specific strains are able to cause systemic infections, i.e., septicemia or endocarditis, in immunologically normal hosts without foreign-body association (3, 28). These strains are reported to possess special virulence factors such as capsules; however, few studies have been published on encapsulated CNS (1, 13, 14, 18).

Encapsulated strains of *Staphylococcus aureus* are more resistant to phagocytosis than nonencapsulated strains (11, 21, 34, 35). Similarly, encapsulated strains of *S. epidermidis* have been found to be resistant to phagocytosis (39).

The present investigation was undertaken to examine the role of capsule on phagocytosis and virulence of *S. simulans*.

**MATERIALS AND METHODS**

**Bacteria.** Encapsulated strain 698 of *S. simulans* was isolated from an intra-abdominal abscess following appendectomy. The unencapsulated strain CCM 2742 of *S. simulans* that was used as a control is maintained in the strain collection of the Institute of Hygiene, University of Cologne. The two strains were identical in cell wall composition, and both produced the following enzymes and toxins: protease, hemolysin, lipase, esterase, and DNase. Capsule is identified by the presence of a halo in India ink preparations (6). A halo was noted with *S. simulans* 698 but not with strain CCM 2742.

**Animals.** Female mice weighing approximately 15 to 18 g (4 weeks old) were inoculated intraperitoneally with *S. simulans*. New Zealand rabbits from the Central Institute for Experimental Animals (Hanover, Federal Republic of Germany) were inoculated intravenously to raise antibodies to *S. simulans*.

**S. simulans vaccine.** *S. simulans* 698 was suspended to a concentration of 10⁸ CFU/ml and heat killed, and 0.5 ml of this suspension was injected into the marginal ear veins of rabbits three times a week for 6 weeks. Ten days after the final inoculation, the rabbits were exsanguinated, and sera were separated and stored. Antibody titers were determined by bacterial agglutination.

**S. simulans capsular material.** *S. simulans* 698 was incubated for 20 h at 37°C in chemically defined medium (30). After centrifugation (25,000 × g for 20 min), the bacterial pellet was suspended in 0.15 M phosphate-buffered saline (pH 7.2) and treated with sonic oscillation (Sonifier 13 12; Branson Sonic Power Co.) for 10 min at 4°C.

After centrifugation (20,000 × g for 20 min at 4°C), the supernatant was filtered (0.45-μm-pore-size membrane filter; Millipore Corp., Bedford, Mass.), dialyzed with distilled water for 48 h at 4°C, and lyophilized.

**Bacterial clearance from peritoneal cavities and heart blood of mice.** *S. simulans* 698 and CCM 2742 were incubated on sheep blood agar for 20 h at 37°C, harvested, and washed twice with sterile saline. Bacterial suspensions were adjusted to an optical density of 1.2 at 580 nm by a model 2.5 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The number of viable bacteria was determined by the number of colonies in appropriate 10-fold dilutions of bacterial suspension inoculated on blood agar and incubated overnight at 37°C. Bacterial suspension (0.5 ml) in saline containing 7.0 × 10⁷ CFU of strain 698 per ml and 9.0 × 10⁷ CFU of strain CCM 2742 per ml was injected into the peritoneal cavities of mice (groups of 25). Five mice in each group were killed at 2, 6, 12, 24, and 48 h after inoculation, and the number of CFU in the peritoneal cavity and heart blood was determined. A total of 0.5 ml of heart blood per mouse was cultured, and peritoneal cavities were lavaged with 3 ml of sterile saline, which was then cultured. The colonies were counted, and results were expressed as the

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total number of organisms in the peritoneal cavity and heart blood.

**Absorbed antisera.** Rabbit antisera against encapsulated strain 698 were absorbed with whole bacterial cells or with capsular material. Rabbit antiserum (1 ml) was incubated with 5 mg of lyophilized heat-killed whole cells or capsular material at 37°C for 1 h and at 4°C overnight. After incubation, the sera were filtered (Millipore filter, 0.45-μm pore size). Sera were heat inactivated by being incubated at 56°C for 30 min.

**Passive immunization with rabbit antiserum.** Doses (0.5 ml) of rabbit antiserum or absorbed antiserum were injected intraperitoneally into mice. Thirty minutes later, the mice were challenged with encapsulated strain 698 (0.5 ml, 5 × 10^7 CFU/ml of saline). After 2 h, the mice were killed and the number of viable bacteria in peritoneal fluid and heart blood was determined as described above.

**Preparation of PMNs.** Human polymorphonuclear leukocytes (PMNs) were prepared for the bacterial killing assay by using the modified test of Van Furth et al. (31). Heparinized blood from a healthy volunteer was mixed with 6% (wt/vol) dextran T2000 (Pharmacia, Uppsala, Sweden; blood to dextran ratio, 4:1 [vol/vol]) and incubated at 37°C for 20 min. PMNs were collected from the leucocyte-enriched plasma layer by centrifugation at 200 × g for 10 min. Erythrocytes were removed by hypotonic lysis with 0.2% (wt/vol) NaCl for 45 s followed by the restoration of isotonicity by the addition of an equal amount of 1.6% (wt/vol) NaCl. After centrifugation at 200 × g for 10 min, the PMNs were washed with Hanks balanced salt solution (without phenol red; GIBCO Ltd., Glasgow, Scotland). PMNs were suspended in RPMI 1640 (GIBCO) containing 10% (vol/vol) fetal calf serum at 10^7 cells per ml. This preparation contained 85% PMNs.

**Bacterial killing assay.** Bacterial killing activity of PMNs was measured by the techniques of Metcalf et al. (22) modified as follows. *S. simulans* 698 and CCM 2742 were incubated on sheep blood agar plates at 37°C for 20 h, harvested, and washed twice with sterile saline. The amount of each strain was adjusted to approximately 5 × 10^7 CFU/ml. Both *S. simulans* strains were opsonized by incubation at 37°C for 30 min in RPMI 1640 containing 10% (vol/vol) normal pooled human serum, rabbit antiserum, or absorbed rabbit antiserum. After opsonization, bacteria were washed twice with sterile phosphate-buffered saline and resuspended in RPMI 1640 containing 10% (vol/vol) fetal calf serum to a concentration of approximately 10^7 CFU/ml. Bacterial suspension (0.1 ml; 10^7 or 10^6 CFU/ml) was added to 1.0 ml of PMN suspension (10^6 cells per ml) and incubated at 37°C for 60 or 120 min with gentle shaking. After incubation, 0.1 ml of each reaction mixture was added to 9.9 ml of distilled water, mixed vigorously and incubated at room temperature for 20 min. The number of viable bacteria was determined.

**RESULTS**

**Bacterial clearance of *S. simulans* from peritoneal cavities and heart blood of mice.** A large capsule was detected on *S. simulans* 698 (Fig. 1). Mice were inoculated intraperitoneally with the strains, and the kinetics of clearance of bacteria from the peritoneal cavity was determined at 2-h intervals. The number of viable bacteria in the peritoneal cavity was unchanged during the first 6 h in mice inoculated with encapsulated strain 698, but more than 95% of strain 2742 was already cleared in 2 h (Fig. 2A).

When heart blood of mice inoculated intraperitoneally was cultured, more colonies of *S. simulans* 698 than of the nonencapsulated strain of *S. simulans* were found. A 4-log difference between the two strains was noted after 2 h. Although strain 698 persisted for longer than 12 h, strain 2742 was cleared from the blood within 12 h (Fig. 2B). However, none of the mice infected with either strain died.

After passive immunization of mice with rabbit antiserum (intraperitoneal application) against encapsulated strain 698, a 99% reduction in organisms in the peritoneal fluid was noted, and only a very small number of organisms were recovered from the blood (Fig. 3A). The protective effect of antiserum was removed by absorption of the antiserum with heat-killed whole cells of *S. simulans* 698 (Fig. 3B). Absorption with *S. simulans* capsular material reduced the protective effect to some extent but was not statistically significant (Fig. 3C).

**Phagocytosis and killing of *S. simulans* by human PMNs.** Phagocytosis and killing of *S. simulans* 698 and CCM 2742 were examined in vitro by using human PMNs and pooled human serum for opsonization. More than 80% of nonencapsulated *S. simulans* CCM 2742 were phagocytized and killed within 2 h by phagocytic mixtures containing PMNs (Fig. 4A). Little phagocytosis or killing of encapsulated strain 698 was observed during 2 h of incubation with PMNs, even when the ratio of PMNs to bacteria was increased from 1:1 to 10:1 (Fig. 4B).

However, encapsulated *S. simulans* 698 was rapidly phagocytized and killed by PMNs after opsonization with rabbit antiserum against *S. simulans* capsule (Fig. 5A). More than 90% of the inoculated bacteria were killed by PMNs within 1 h. Opsonic activity of the rabbit antiserum was removed to a great extent by absorption of the antiserum with heat-killed whole cells (Fig. 5B) or with capsular material prepared from encapsulated strain 698 (Fig. 5C).
OHSHIMA et al.

FIG. 2. Clearance of nonencapsulated strain CCM 2742 and encapsulated strain 698 of S. simulans in peritoneal fluid (A) and heart blood (B) of mice. Results are shown as averages of viable number of bacteria from each group of five mice at 2-h intervals.

DISCUSSION

CNS are recognized as significant nosocomial pathogens despite being normal inhabitants of human skin and mucosal membranes (2, 4, 7, 14, 19, 29, 37). The presence of slime is strongly correlated with these CNS infections (8, 9, 24, 25). Several investigators have reported that some CNS strains isolated from clinical specimens produce capsular material (1, 13, 14, 17, 18, 40). These encapsulated strains of CNS show higher resistance to phagocytosis than do unencapsulated strains (39). Additionally, the capsular substances from S. aureus and S. epidermidis contribute to increased virulence in experimental infections with these microorganisms (11, 18, 21, 39).

We have compared an encapsulated and an unencapsulated S. simulans strain identical in cell wall compositions and enzymatic properties. Our experimental results suggest that the surface capsule of S. simulans had a major anti-phagocytic effect. The capsule appeared to inhibit interaction of bacterial surfaces with opsonins such as complement and antibacterial antibodies which promote phagocytosis and killing of bacteria by phagocytic cells. The role of the capsule, therefore, is similar in this CNS species and in S. aureus and other bacterial species (1, 35, 38).

Cell wall peptidoglycan of S. aureus plays a key role in resistance to phagocytosis, but opsonization of the cell wall by complement activated via the alternative pathway provides a natural host defense mechanism (27, 32, 35). However, strains of S. aureus which are encapsulated are not opsonized by complement in serum without specific antibodies (26, 35). Therefore, specific antibodies against capsular antigens are required for effective opsonization of encapsulated S. aureus, and results from this study suggest that they are necessary for opsonization of encapsulated S. simulans as well.

After intraperitoneal inoculation of mice with encapsulated S. simulans, rapid invasion and persistent colonization of the bloodstream was observed. Large numbers of organisms also persisted in the peritoneal cavity. Despite this, none of the infected mice developed disease or died. After passive immunization with rabbit antiserum containing anti-capsular antibodies, however, no differences between encapsulated and nonencapsulated strains of S. simulans were
observed, suggesting that anticapsular antibodies are opsonic, allowing rapid clearing of intraperitoneal organisms, and prevent bloodstream invasion. Specific antibodies against capsular material, therefore, appear to be necessary for host defense against encapsulated strains of CNS.

Opsonic binding of the C3 molecule of complement to bacterial surfaces appears to be inhibited by the presence of a capsule (18, 34, 38). Heat inactivation of the S. simulans antiserum (56°C, 30 min) does not diminish its effectiveness as an opsonin for encapsulated S. simulans, suggesting that these organisms are effectively opsonized by specific antibodies and that enhanced opsonization by complement cannot be detected (5, 16). Absorption of these antisera with S. simulans capsular substance decreased opsonic activity, providing strong evidence that it was anticapsular antibodies which provide effective opsonin for encapsulated S. simulans, promoting rapid uptake and killing by human PMNs.

Bacterial cell wall peptidoglycan of encapsulated S. simulans may bind complement on the cell wall surface, as previously demonstrated with S. aureus (26). However, opsonic or ligand activity, i.e., binding of bacteria to phagocytic cell surface, is blocked by the capsule (5, 16). Recently, we demonstrated that the S. simulans capsule also acts as a barrier for the interaction of a polyvalent staphylococcal bacteriophage with its receptor in the S. simulans cell wall (23).

The presence of capsule on the surface of S. simulans appeared to play a role in resistance to phagocytosis; however, this property may be associated with other virulence factors in vivo.

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LITERATURE CITED


