Relation of Mucoid Growth of *Staphylococcus aureus* to Clumping Factor Reaction, Morphology in Serum-Soft Agar, and Virulence

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The growth characteristics of several strains of *Staphylococcus aureus* in Brain Heart Infusion and in a modified Staphylococcus Medium No. 110 were compared. In the latter medium all of the strains studied showed an increased mucoid character. Some of the strains studied showed a greater potential to synthesize excess slime layer material than others. The highly mucoid strains grew as diffuse-type colonies in modified Staphylococcus Medium No. 110 serum-soft agar and reacted as though they were negative in the test for clumping factor. These strains were also found to be more virulent when used to challenge normal mice intraperitoneally.

One of the most discouraging aspects of research in the field of infection and resistance to *Staphylococcus aureus* has been the lack of a suitable laboratory experimental system. Since Hunt and Moses (7) described the greater mouse virulence of the diffuse variant of the Smith strain of *S. aureus*, several investigators have used this organism, or a strain with similar properties, to study various aspects of experimental staphylococcal disease (1, 5, 6, 10, 13, 15). Our own studies with the Smith diffuse strain of *S. aureus* (2, 3) suggested that, in addition to the "Smith Surface Antigen" described by Morse (11), teichoic acid from this strain also shows in vitro immunological properties and is an important protection-inducing antigen in mice.

In an extension of these studies to other non-Smith-like strains of staphylococci, we were again faced with the problem of virulence of these organisms for mice. In this paper, we report a method which has been successful in enhancing the mouse virulence of several strains of *S. aureus* and in inducing them to exhibit growth characteristics similar to the Smith diffuse-type strains.

**Materials and Methods**

**Organism.** The diffuse and compact variants of the Smith strain and of the SK4473 strain were obtained from M. C. Koenig, Vanderbilt University School of Medicine, Nashville, Tenn. The 258, 334, and 418 strains were obtained from M. Silverman, Veteran's Administration Research Hospital, Chicago, Ill., as recent clinical isolates. The MD strain was isolated from an abscess in a dog, and the *S. aureus* type I strain was obtained from the American Type Culture Collection. All strains were positive in the test for clumping factor, with the exception of the Smith diffuse strain. They were all positive for free coagulase in the tube test and were hemolytic on rabbit blood-agar.

**Culture medium.** In addition to the routine use of Brain Heart Infusion (BHI) broth (Difco) prepared as suggested by the manufacturer, a modified dialysate medium similar in its composition to Staphylococcus Medium No. 110 (Difco) was prepared as follows: 2.5 g of yeast extract (Oxoid) and 10 g of peptone (Difco) were dissolved in 50 ml of 3.0% NaCl and placed into a dialysis bag. The bag was dialyzed against 500 ml of 3.0% NaCl for 2 days at 4°C with a magnetic stirrer. To the dialysate we then added 10 g of mannitol, 2 g of lactose, and 5 g of K2HPO4. Agar (Difco), to a concentration of 1.5%, was added to a portion of this medium, and the remainder was prepared as a broth. In preliminary experiments, we found that better growth is obtained when the agar medium is supplemented with additional buffer. Therefore, before sterilization, phosphate buffer (pH 7.4) was added to the modified 110 agar to a final concentration of 0.05 M. The media were autoclaved at 121°C for 10 min and were stored at 4°C until used.

**Preparation of cell suspensions and challenge method.** The organisms used in these studies were maintained by monthly transfer on BHI agar slants. To prepare standardized cell suspensions for challenge experiments, the organisms were subcultured into modified 110 broth and were incubated at 37°C for 18 hr. A 5-ml amount of these cultures was then pipetted onto the surface of medium 110 agar plates containing approximately 30 ml of medium. The plates were incu-
bated for an additional 18 hr at 37 C. After incubation, the excess broth was gently discarded and the organisms were carefully washed three times with cold fresh media while still on the plates. The organisms were then scraped into small tubes containing cold fresh media and were allowed to settle for 10 to 15 min at room temperature. The supernatant fluids were discarded, and cold fresh medium was added. The organisms were then dispersed with an inoculating loop. The cell suspensions were again allowed to settle for 15 min at 4 C to remove large clumps. The organisms from the upper part of suspensions were then adjusted turbidimetrically at 430 nm in a Coleman model 9 nephlocolorimeter, with the fresh modified 110 broth as a blank. Plate counts of appropriate dilutions of these suspensions showed that an optical density (OD) of 0.5 corresponded to 1.8 x 10^6 to 3.3 x 10^6 colony-forming units (CFU) per ml. Ten times concentrated suspensions were prepared by centrifuging the 0.5 OD cell suspensions and resuspending them in 0.1 volume. Ten-fold dilutions were made from the 0.5 OD cell suspensions with fresh modified 110 broth. Organisms grown on BHI agar plates were used as a control. A 5-ml amount of BHI broth cultures was transferred onto BHI agar plates containing 30 ml of BHI agar and was incubated at 37 C for 18 hr. The organisms were washed with cold modified 110 broth three times, and the 0.5 OD cell suspensions were prepared and diluted in cold fresh modified 110 medium as described above. Plate counts of these suspensions varied from 1.6 x 10^9 to 4.5 x 10^9 CFU/ml. A 0.5-ml amount of each cell suspension was injected intraperitoneally into groups of adult (20 to 25 g) CF1 mice (Carworth Farms, New City, N.Y.) immediately after preparation. After challenge, the animals were observed and deaths were recorded for a period of 1 week; then the experiments were terminated.

To determine the direct toxicity of the surface slime material elaborated by the organisms grown on modified 110 agar, a concentrated preparation of this material was made in the following way. Organisms grown on modified 110 agar for 18 hr at 37 C were harvested in 110 broth. The cells were separated by centrifugation at 15,000 x g for 20 min, resuspended in 20 ml of distilled water, and stirred in a Waring Blender for 10 min at room temperature. The cells were again separated as described above, and the supernatant fluid was dialyzed against distilled water and lyophilized. Several concentrations of the lyophilized material, dissolved in fresh modified 110 medium, were injected intraperitoneally into groups of normal mice.

Serum-soft agar was prepared with either BHI or modified 110 broth according to the method of Finkelman and Sulkin (4); 1% normal rabbit serum and 0.15% agar (Difco) were used. The clumping factor (CF) reaction was determined by standard methods with undiluted normal rabbit plasma.

**RESULTS**

**Growth characteristics of the strains of S. aureus in modified 110 and BHI media.** All of the organisms studied showed a strikingly increased mucoid or viscous type of growth on modified 110 agar as compared with organisms grown on BHI agar. Figure 1 shows cultures of the MD strain of S. aureus on modified 110 agar and on BHI agar. The viscosity and excess slime production is evident. In modified 110 broth, the organisms also showed a viscous type of growth, with most of the organisms settling to the bottom of the tubes during incubation. When the tubes were swirled, a rosy mass of organisms rose in the tubes but did not disperse. In BHI broth, there was some settling of the organisms but they could be dispersed easily into a homologous cell suspension. Figure 2 shows a phase-contrast photomicrograph of the MD strain grown on modified 110 agar. The marked slime layer present around the organisms could be observed either in moist or in dried India-ink preparations, but were more clearly seen in the latter. Nothing even remotely resembling this type of material could be observed with the organisms grown on BHI agar.

All of the organisms studied, with the exception of the Smith diffuse strain of S. aureus, showed a characteristic "compact" type of colonial morphology when grown in BHI serum-soft agar. In striking contrast, however, the Smith compact, SK4473, and MD strains showed "diffuse" type growth when cultured in modified 110 serum-soft agar. With the S. aureus type I, 258, 334, and 418 strains, however, most of the colonies showed an intermediate type of growth, between a typical diffuse and typical compact colony.

Figure 3a shows the Smith compact strain growing in BHI and 110 serum-soft agar, and Fig. 3b shows the 258 strain growing under the same conditions. When the diffuse-growing mutants from the 258 cultures were carefully isolated in a capillary pipette and subcultured into fresh 110 serum-soft agar, all of the colonies showed the typical diffuse type of morphology. On the other hand, when diffuse-growing organisms in 110 medium were transferred to BHI serum-soft agar, all grew with the compact type of colonial morphology.

**Virulence of organisms grown on modified 110 media.** A comparison was made of the mouse virulence of the staphylococcal strains studied after cultivation for 18 hr on BHI or modified 110 agar plates. In preliminary experiments, we found that routine washing of the mucoid organisms with saline removed much of the slime layer material and reduced the virulence of the organisms. Therefore, these organisms were carefully washed as described in Materials and Methods, and were suspended in fresh 110 medium for injection. The organisms cultured on BHI agar...
Fig. 1. MD strain of S. aureus grown on BHI or modified Staphylococcus Medium No. 110 agar. Plates were inoculated with 3 to 5 ml of broth culture, incubated at 37 C for 18 hr, and agitated before the photograph was taken.

Fig. 2. India ink phase-contrast photomicrograph of MD strain of S. aureus grown on modified Staphylococcus Medium No. 110 agar.
were handled in a similar manner and were suspended in fresh 110 medium for injection. To confirm the injection of comparable challenge doses, plate counts of these suspensions were always done.

The three strains of *S. aureus* (Smith compact, SK4473, and MD) which could be induced to grow as typical diffuse-type colonies in modified 110 serum-soft agar also showed an enhanced virulence in mice, whereas those strains (258, 334, 418, and type I) which could not be induced to exhibit typical diffuse-type growth under these conditions did not show a significant enhancement of their virulence for mice (Table 1).

Since the organisms used in these studies were grown in modified 110 medium and were prepared for injection in 110 broth, it was necessary to determine whether the media had any adverse effect on the animals. Since the modified 110 broth contains 3.0% NaCl, it was considered possible that this hypertonic salt solution might decrease the resistance of the mice by virtue of its effect on the viability or phagocytic activity of peritoneal leukocytes. In control experiments, however, the same organisms cultured on BHI agar and prepared for injection in 110 broth did not show an increase in virulence. In addition, we found that normal mice injected intraperitoneally with as much as 25 mg of lyophilized slime material dissolved in modified 110 broth showed no ill effects and survived indefinitely.

**Intraperitoneal phagocytosis of mucoid and nonmucoid strains.** Mucoid and nonmucoid organisms of the same strain were injected into separate groups of normal mice at a dose of approximately $10^8$ CFU in 0.5 ml of 110 broth. Within 5 min of the injection and at 3 hr, groups of three mice were sacrificed and their peritoneal cavities were washed out with 1.0 ml of sterile saline. With a modified Maaløe procedure as described by Koenig (8), the total number of organisms recoverable, those extracellular, and those associated with phagocytic cells were determined by differential centrifugation and plating. Figure 4

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**Fig. 3.** Colonial morphology of (a) Smith compact and (b) 258 strains of *S. aureus* in BHI and modified Staphylococcus Medium no. 110 serum-soft agar.
shows the results of a typical experiment of this kind with the MD strain. It is clear that the mucoid organisms were much less efficiently handled than the nonmucoid organisms under the conditions of these experiments.

Effect of washing on virulence and reactivity in the test for clumping factor. Table 2 shows the results of (CF) tests with organisms grown on modified 110 and BHI agar, and the effect of washing the organisms on their reaction in these tests. In these experiments, the mucoid organisms were washed by repeatedly centrifuging them from homogenous saline suspensions; this procedure should be distinguished from the careful preparation of cell suspensions for the challenge experiments as described in Materials and Methods. It is clear that organisms grown on modified 110 agar react as though they are CF negative before washing, but became CF positive after 1 to 2 saline washes. Table 3 shows the effect of similar washing experiments on the virulence of the MD and 258 strains grown on modified 110 agar. After each wash, a sample of the organisms was adjusted turbidimetrically to contain approximately 10^6 CFU per ml in the case of the MD strain and 10^6 CFU per ml in the case of the less mucoid 258 strain; 0.5 ml was injected intraperitoneally into groups of mice. It is clear that repeated washing, presumably by removing the slime layer material, reduced the virulence of the organisms. The Smith diffuse strain of *S. aureus* remained CF negative and virulent through repeated washes.

It was also shown that BHI-grown organisms suspended in a 5% saline solution of crude homologous slime layer material are not more virulent than the same organisms suspended in saline. These experiments were done to investigate the possibility that the slime layer was functioning in a manner similar to mucin (3).

**DISCUSSION**

For many years, a number of investigators (12) have engaged in the controversy regarding the existence of true capsules in the genus *Staphylococcus*. It is generally conceded that the Smith strains of *S. aureus*, as described by Koenig and Melly (9), can be shown to possess true

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**TABLE 1.** Mouse virulence of *S. aureus* strains grown on modified *Staphylococcus medium No. 110 or Brain Heart Infusion agar**

<table>
<thead>
<tr>
<th>Challenge dose (CFU)</th>
<th>Smith compact BHI</th>
<th>SK4473 BHI</th>
<th>MD 110 BHI</th>
<th>Type I 110 BHI</th>
<th>258 110 BHI</th>
<th>334 110 BHI</th>
<th>481 110 BHI</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^8</td>
<td>10^10</td>
<td>6/10</td>
<td>10/10</td>
<td>10/10</td>
<td>9/10</td>
<td>9/10</td>
<td>9/10</td>
</tr>
<tr>
<td>10^7</td>
<td>15/20</td>
<td>1/15</td>
<td>10/10</td>
<td>7/10</td>
<td>8/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>10^6</td>
<td>1/10</td>
<td>0/10</td>
<td>6/10</td>
<td>0/10</td>
<td>2/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>10^5</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* No. dead/no. challenged.

**TABLE 2.** Effect of washing strains of *S. aureus* grown on modified *Staphylococcus Medium No. 110 or Brain Heart Infusion (BHI) agar on their reaction in the test for clumping factor

<table>
<thead>
<tr>
<th>Strain of <em>S. aureus</em></th>
<th>Grown in</th>
<th>Clumping factor reaction before wash</th>
<th>Washed once</th>
<th>Washed twice</th>
<th>Washed three times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smith diffuse</td>
<td>BHI</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Smith compact</td>
<td>BHI +</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>110 -</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MD</td>
<td>BHI +</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>110 -</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>258</td>
<td>BHI +</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>110 -</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Clumping factor reaction absent, -; clumping factor reaction present, +; ND, not done.
Table 3. Effect of washing the 258 and MD strains of S. aureus grown on modified Staphylococcus medium No. 110 agar on their mouse virulence

<table>
<thead>
<tr>
<th>Times washed</th>
<th>Mortality after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>258a strain</td>
</tr>
<tr>
<td>None</td>
<td>16/25b</td>
</tr>
<tr>
<td>Once</td>
<td>1/20</td>
</tr>
<tr>
<td>Twice</td>
<td>0/20</td>
</tr>
<tr>
<td>Three times</td>
<td>4/10</td>
</tr>
</tbody>
</table>

a The 258 strain was injected intraperitoneally at a dose of approximately 10⁶ CFU per mouse, whereas the more mucoid MD strain was injected at a dose of approximately 10⁷ CFU per mouse.

b No. dead/no. challenged.

capsules; recently, Wiley and Mavarakis (17) were able to demonstrate a “specific capsular reaction” with these organisms in homologous antiserum. The “wound strains” of S. aureus described by Wiley (16) also appear to elaborate capsular material and have been shown to undergo a specific capsular reaction in homologous antiserum; Mudd (12) has suggested that this phenomenon be designated the extracellular peripheral precipitation reaction.

Whereas the Wiley wound strains were isolated after prolonged incubation in a glycerol-broth medium and probably represent the selection of mutant organisms, the phenomenon described in this paper involved the induction of mucoid growth in a medium containing lactose, mannitol, and NaCl and similar in its composition to Staphylococcus medium No. 110. The conversion from nonmucoid to mucoid organisms occurred in various degrees after the initial growth of all strains studied in this medium. In addition, subculture back into BHI media resulted in a complete return to the nonmucoid form. These results are similar to the pseudocapsulation reaction described by Sall, Mudd, and Taubler (14), except that gelatin was not required in our medium and at least 3% NaCl was essential.

The three strains, Smith compact, SK4473, and MD, showed the most striking alteration to mucoid growth on modified 110 agar, and these strains converted completely to diffuse-type growth in 110 serum-soft agar. On the other hand, the type 1, 258, 334, and 481 strains were less mucoid on 110 agar and showed only a few diffuse colonies in 110 serum-soft agar; these strains were intermediate in character or remained as compact colonies under these conditions.

While the mucoid strains resembled the typical Smith diffuse strain in reacting as though they were negative for CF, growing as diffuse colonies in 110 serum-soft agar, and showing increased mouse virulence, it should be made clear that the similarity was related solely to the synthesis of excess slime layer by these organisms in response to the nutritional conditions provided by the modified 110 medium. The slime layer could be removed by several saline washes; in so doing, the organisms became CF positive and less virulent, suggesting that the slime layer merely covered the CF on the cell surface and also made the organisms less susceptible to phagocytosis in vivo.

It was of interest to observe, however, that different strains varied in their potential to elaborate large amounts of slime layer under these conditions; by utilizing the serum-soft agar technique with modified 110 medium, those organisms in a mixed population which had the greatest potential in this regard could be distinguished and selected for.

Isolated crude slime layer material was shown to be nontoxic in milligram amounts, and preliminary analyses indicate that it is primarily polysaccharide in nature. Further studies on the chemical, immunochemical, and protection-inducing properties of the slime layer material are in progress.

Finally, in preliminary experiments the diffuse-growing mutant organisms isolated from the 258 strain by the modified 110 serum-soft agar selection technique were shown to be more virulent than organisms from the mixed culture. Further experiments are in progress to confirm this observation and to determine whether it occurs in a group of strains recently isolated from clinical material.

Acknowledgment

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Literature Cited


