Nonproduction of Toxic Shock Syndrome Toxin 1 by Coagulase-Negative Staphylococci

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We tested 187 strains of coagulase-negative staphylococci (CNS) for the production of toxic shock syndrome toxin 1 (TSST-1). A total of 111 CNS strains were isolated from the tampons of menstruating women and 74 were isolated from unused tampons. Two strains were isolated from the genital tract of a patient with toxic shock syndrome. Strains were cultured by the membrane-over-agar method to enhance production of TSST-1, and culture supernatants were tested by two exquisitely sensitive enzyme-linked immunosorbent assays. None of the 187 CNS strains produced TSST-1. We conclude that CNS colonizing the genital tracts of menstruating women and unused tampons produce TSST-1 infrequently, if ever, and are unlikely to play a role in toxic shock syndrome.

Toxic shock syndrome (TSS) is caused by a toxin or toxins produced by Staphylococcus aureus. The only specific toxin identified to date is TSS toxin 1 (TSST-1), but it is clear that other staphylococcal exoproducts, possibly including the enterotoxins, may also serve as etiologic agents (2-4, 13). Regardless of the clinical setting, virtually all patients with TSS are colonized or infected with S. aureus (14), although recovery of this organism is not requisite for diagnosing TSS. Recently, however, Crass and Bergdoll reported seven patients with TSS from whom only TSST-1-producing, coagulase-negative staphylococci (CNS) were isolated (1). In addition, toxin-producing CNS from a variety of other clinical settings were identified, as well as several strains from unused tampons.

Because of the frequency with which women are colonized by CNS (7), a growing appreciation of the potential pathogenicity of these strains (8, 12), and the reported isolation of CNS from unused catamenial products (1), we felt it was important to confirm the findings of Crass and Bergdoll by studying toxin production by CNS prospectively. We report here our data on toxin production by CNS isolated from unused tampons and from the tampons of menstruating women. In addition we tested two CNS strains, previously reported to produce TSST-1, isolated from a patient with TSS (6).

MATERIALS AND METHODS

CNS from menstruating women. CNS strains were isolated from the tampons of healthy, menstruating women. Hospital employees from several Harvard University-affiliated hospitals were asked to submit tampons that had been in place for 4 to 6 h, as part of a study primarily designed to identify women who are colonized by TSST-1-producing S. aureus. Our methods allowed for the isolation of CNS as well as S. aureus. Tampons were rolled onto sheep blood agar and mannitol salt agar plates, and the plates were streaked and then incubated at 37°C overnight. Our initial procedure was to subculture colonies that demonstrated robust growth on mannitol salt agar at 24 h. S. epidermidis appeared to be underrepresented by this method. Subsequently, therefore, colonies showing morphology typical of staphylococci on blood agar or on mannitol salt agar at 48 h were subcultured as well. One catalase-positive, gram-positive coccus, demonstrating the predominant colonial morphology on the plates, was saved from each tampon for further analysis.

CNS from unused tampons. CNS strains were isolated from unused tampons purchased from local retailers. Multiple styles of the following brands were cultured: Kotex (Kimberly-Clark Corp.), o.b. (Personal Products Co.), Playtex (International Playtex, Inc.), Tampax (Tambrands Inc.), and Pursettes (Jeffrey Martin, Inc.). Sterile technique was used to prevent contamination by extraneous staphylococci. Tampons were removed from their wrappers and placed in 50-ml centrifuge tubes, to which was added 30 ml of medium. A restrictive medium, consisting of brain heart infusion, 6.5% sodium chloride, and 0.003% sodium azide, was used to suppress the multiplication of most other bacteria (15). Cultures were incubated at 37°C and subcultured onto blood agar plates at 24 and 48 h. All catalase-positive, gram-positive cocci from each tampon showing different colonial morphologies were saved for subsequent analysis.

CNS from a patient with TSS. Two strains of CNS isolated from a patient with TSS were provided by Ralph C. Kahler, University of Mississippi Medical Center. These strains were previously reported to be producers of TSST-1 (1, 6).

Identification of CNS species. Strains were tested for the production of staphylococcal coagulase by two methods. All strains were tested by the Staphaurex kit (Wellcome Diagnostics, Dartford, England), which identifies S. aureus on the basis of agglutination of latex particles coated with immunoglobulin G and fibrinogen. All positive strains and strains demonstrating equivocal results by Staphaurex were tested for coagulase production by the tube method. Overnight cultures (0.1 ml) of staphylococci in brain heart infusion broth were added to 0.5 ml of coagulase plasma (BBL Microbiology Systems, Cockeysville, Md.) and incubated at

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TABLE 1. CNS isolated from tampons of menstruating women and from unused tampons

<table>
<thead>
<tr>
<th>Staphylococcus species</th>
<th>No. (%) isolated</th>
<th>Used tampons</th>
<th>Unused tampons</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. hemolyticus</em></td>
<td>62 (56)</td>
<td>7 (10)</td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>24 (22)</td>
<td>48 (65)</td>
<td></td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>8 (7)</td>
<td>4 (5)</td>
<td></td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>5 (4)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>5 (4)</td>
<td>4 (5)</td>
<td></td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>3 (3)</td>
<td>11 (15)</td>
<td></td>
</tr>
<tr>
<td><em>S. capitis</em></td>
<td>2 (2)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>1 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. colnii</em></td>
<td>1 (1)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

37°C. Strains demonstrating coagulase activity at 1, 4, or 24 h were excluded from further analysis.

To further assure that strains of *S. aureus* were not being misidentified as CNS, all isolates were identified to species by one or both of two methods: the Staph-Ident system (API Analytab Products, Plainview, N.Y.) and the AMS AutoMicrobic system (Vitek Systems Inc., Havelwood, Mo.). All strains were tested by Staph-Ident; strains showing equivocal results were tested in the AMS system, as were a number of randomly chosen strains. Novobiocin disks (Scott, Fiskeville, R.I.) were used to differentiate between *S. saprophyticus* and *S. hominis* when Staph-Ident was used.

Production of and assay for TSST-1. CNS were cultivated by a modified membrane-over-agar method (5) to maximize production of TSST-1. Brain heart infusion agar was overlaid with autoclaved dialysis membrane (molecular weight cutoff, 12,000 to 14,000) (Spectrum Medical Industries Inc., Los Angeles, Calif.), and bacteria were inoculated into the membrane. After overnight incubation at 37°C, the bacteria were harvested in a small volume of saline and centrifuged, and the supernatant was tested for TSST-1.

A competitive enzyme-linked immunosorbent assay (ELISA) (11) was used to test for the production of TSST-1. This assay uses a TSST-1–alkaline phosphatase conjugate, made from TSST-1 purified as described previously (10). The assay has a lower limit of quantitation of 0.001 μg/ml. CNS supernatants are run at a dilution of 1:30 to avoid nonspecific interference, yielding a final sensitivity of 0.03 μg/ml. TSST-1-producing strains of *S. aureus* generally yielded >2.0 μg of TSST-1 per ml when cultivated and tested by these methods.

Some strains were also tested in a sandwich ELISA, as indicated below. Microtiter plates were coated with polyclonal rabbit antiserum to TSST-1. TSST-1-containing samples and standards were added, followed after washing by a monoclonal antibody to TSST-1 (provided by Peter Bonventre, University of Cincinnati College of Medicine) and then a goat anti-mouse immunoglobulin G alkaline phosphatase conjugate (Cooper Biomedical, Inc., West Chester, Pa.). This assay provided a lower detection limit of 0.0002 μg/ml; samples were run at a 1:10 dilution, yielding a final sensitivity of 0.002 μg/ml.

RESULTS

CNS from tampons of menstruating women. By the methods described, it was possible to isolate at least one staphylococcal strain from most participants in our study. Coagulase-positive strains have been isolated from 48 of 368 (13%) women cultured to date; 9 (19%) of these strains produce TSST-1. These strains will be described elsewhere.

The first 111 CNS isolated from the tampons of menstruating women were saved, identified to species, and tested for production of TSST-1. Table 1 reveals that *S. hemolyticus* was the most commonly identified species (56%), followed by *S. epidermidis* (22%). The true frequency of colonization by *S. epidermidis* is probably higher, being underrepresented here because of its slower growth on manniitol salt agar, as noted above.

No CNS were found to produce TSST-1. Known TSST-1-producing strains of *S. aureus* were cultivated and tested concurrently with each batch of CNS, generally yielding TSST-1 concentrations of >2.0 μg/ml. The competitive ELISA yielded a definitive result of <0.03 μg/ml for 97 of 111 strains; the 14 remaining strains made 0.03 to 0.08 μg of TSST-1 per ml according to this assay. When tested in the monoclonal ELISA, however, all 14 CNS were definitively negative for TSST-1, producing <0.002 μg/ml. We believe that the low level of positivity demonstrated by these strains with our competitive method was due to nonspecific interference by other staphylococcal products with the binding of our TSST-1 conjugate to the sensitized plate. Of the 14 false-positive strains, 13 were *S. epidermidis*, suggesting that staphylococcal slime may have been the offending substance.

Because we evaluated only one CNS from each tampon, it was not possible to correlate colonization by a particular CNS with the type of tampon worn.

CNS from unused tampons. A total of 151 unused tampons of multiple styles, the products of five manufacturers and representing 24 different lots, were cultured as described. One or more CNS were recovered from 55 tampons (36%), yielding a total of 74 different isolates. Table 1 reveals that *S. epidermidis* was the most frequently isolated CNS. Tampons made by all five manufacturers grew multiple species of CNS, with *S. epidermidis* the species most frequently isolated from each brand. There were no apparent associations between individual tampon brand and any particular species.

No CNS isolated from unused tampons produced TSST-1. Positive controls were included with each assay and yielded >2.0 μg of TSST-1 per ml. The competitive ELISA gave a definitive result of <0.03 μg/ml for all 74 CNS isolates.

CNS from a patient with TSS. Two CNS isolated from a patient with TSS were cultivated and tested as described above. A vaginal isolate, confirmed as being coagulase negative, was identified as *S. epidermidis* by the API method. A labial isolate, also coagulase negative, was identified as *S. hemolyticus*. Both strains were definitively negative for production of TSST-1 in the competitive ELISA. Although previously reported to be producers of TSST-1 (1, 6), other investigators have found them to be negative for TSST-1 production and not to contain the gene for TSST-1 (P. M. Schlievert and B. Kreiswirth, personal communication).

DISCUSSION

We have isolated 111 CNS from tampons of menstruating women and 74 CNS from unused tampons. We were unable to demonstrate the production of TSST-1 by any of these strains, despite cultivation by a toxin-enhancing method and an ostensibly sensitive assay for TSST-1. In addition, we tested two CNS previously reported to produce TSST-1 and found them to be negative for TSST-1 production. Our study supports the recent observation by Crass and Bergdoll (1) that CNS may produce TSST-1 and thereby be involved in the pathogenesis of TSS.
Methodological differences between the two studies may account for our discrepant results. Crass and Bergdoll called “coagulase negative” those staphylococcal strains that yielded a 1+ result in their coagulase assay, as well as strains that were 2+ coagulase positive with a negative test for thermonuclease. We see no justification for terming weakly positive strains “negative”; furthermore, coagulase and thermonuclease tests agree in most cases (9), so that S. aureus strains misidentified by one method may well be missed by the other. The authors do not state whether their TSST-1-producing CNS are among these weakly coagulase-positive strains, but we see this as a potential source of error in their analysis. We used both a tube coagulase test and the Staphaurex latex agglutination test to identify S. aureus strains, the latter being positive in the presence of either staphylococcal clumping factor or protein A. Furthermore, all coagulase-negative isolates were identified to species by Staph-Ident and many by the AMS AutoMicrosystem, virtually assuring that no S. aureus strain was termed coagulase negative.

Second, Crass and Bergdoll used hyperimmune rabbit serum (presumably polyvalent) and “crude toxin standards” in their immunodiffusion assay for TSST-1. A bacterial product other than TSST-1 present in both the toxin standard and the test supernatant may yield a line of identity against polyvalent serum, resulting in a false-positive determination. We used highly purified TSST-1 in our competitive ELISA, making false-positive results unlikely (11), and confirmed as negative a small number of marginally equivocal strains by using a monoclonal antibody against TSST-1.

The data presented by Crass and Bergdoll appear to have been collected retrospectively, and the results do not seem to have been confirmed by repeated testing with uniform reagents under rigidly controlled conditions. Confirmation of their findings by other investigators would also validate their observation. Unfortunately, these strains have not been disseminated widely and were not made available to us upon request. Furthermore, only one other publication has described the production of TSST-1 by CNS (6), and the testing for TSST-1 was performed by Bergdoll’s laboratory. The strains provided by Kahrilas were among those reported in the earlier publication by Crass and Bergdoll.

Our finding that none of 187 CNS produces TSST-1 does not prove, of course, that CNS cannot produce TSST-1. Crass and Bergdoll relate that “about 10%” of “approximately 2,000” staphylococcal cultures submitted to them have been CNS; of these, 33 produced “one or more toxins” (an unspecified number making TSST-1). Despite differences in how strains were collected by the two laboratories, however, we find it difficult to reconcile their 17% rate of toxin production (33 of about 200) with our rate of 0%, unless major technical differences are responsible.

Resolution of this controversy is critical, given the high rate of colonization of women, and of unused tampons, by CNS and the rising incidence of infection by CNS. Dissemination of allegedly positive strains and examination of other pathogenic and colonizing strains of CNS by other laboratories is the only way to definitively resolve the issue. Meanwhile, we submit that there are many reasons why TSST-1-producing S. aureus might not be identified from a patient with suspected TSS. An etiologic role for staphylococci other than S. aureus is not likely to be among them.

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LITERATURE CITED