Production of Toxic Shock Syndrome-Like Illness in Rabbits by
Staphylococcus aureus D4508: Association with Enterotoxin A

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Staphylococcus aureus D4508, obtained from a patient with nonmenstrual toxic shock syndrome (TSS),
produced enterotoxin A while not making other known enterotoxins or TSS toxin I. Concentrated culture fluids
of the organism, administered subcutaneously in miniosmotic pumps, induced TSS-like symptoms (four of six
animals succumbed). Identical culture fluids pretreated with anti-enterotoxin A serum failed to induce
symptoms except for fever (none of six animals succumbed). Purified staphylococcal enterotoxin A also had the
ability to induce TSS-like symptoms. These data suggest that enterotoxin A is the major TSS-associated toxin
made by strain D4508.

Toxic shock syndrome (TSS) is an acute multisystem illness characterized by fever, hypotension, rash, desquamation
of the skin upon recovery, and a variable multiorgan component (13, 30, 32, 33). Staphylococcus aureus strains
expressing toxins are considered the cause of TSS. Most notably, TSS toxin 1 (TSST-1) is the major toxin associated
with the illness and has been used to induce a TSS-like illness in experimental animals (1, 2, 14, 22, 25, 27). In
addition, recent reports have implicated staphylococcal enterotoxins, mainly types B and C, and group A streptococcal
scarlet fever toxins A, B, and C as additional causes of TSS and TSS-like illnesses (9, 11, 12, 23, 25, 32).

Since 1980 there have been occasional reports of other factors associated with TSS isolates. Cohen and Falkow (10)
identified two proteins with molecular weights of approximately 31,000 as highly associated with TSS, and Kapral
identified an epidermal toxin associated with TSS (18). Recently, Scott et al. (28, 29) reported that a staphylococcal
strain designated D4508 expressed a toxin (TSS toxin 2 [TSST-2]) associated with TSS-like symptoms in rabbits.
This strain was obtained from a patient with symptoms consistent with nonmenstrual TSS (15, 29). Finally, Hall et
al. (17) also reported on the production of a toxin from strain D4508; D4508 does not make TSST-1 (15).

This investigation was undertaken to further characterize the D4508 staphylococcal strain with regard to the produc-
tion of a toxin(s) capable of inducing TSS-like symptoms in rabbits. The data suggest that enterotoxin A is the major
TSS-associated toxin made by that strain.

MATERIALS AND METHODS

Bacteria. S. aureus strains used in this study included D4508 (also known as CDC 11), which was kindly provided
by P. Hayes, Centers for Disease Control, Atlanta Ga.; MN8, which makes TSST-1 (26); MNHO, which expresses
enterotoxin B; MNDON, which produces enterotoxin C1 (8); and FRI 722, which makes enterotoxin A and which was
generously provided by M. S. Bergdoll, Food Research Institute, University of Wisconsin, Madison. The strains
were stored lyophilized at 4°C.

Toxin production. S. aureus strains were cultured until the

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bridized. The gene probe was a 297-base-pair BamHI-HindII internal TSST-1 gene (tst)-specific DNA fragment (5). Hybridization was performed under previously described conditions (19).

**Biological assays.** Alzet miniosmotic pumps (Alza Corp., Palo Alto, Calif.) containing either toxins or culture fluids with toxins were implanted subcutaneously in the flanks of American Dutch belted rabbits (22). The animals, which weighed 1 to 2.0 kg, were monitored for 7 days for TSS symptoms as described by Parsonnet et al. (22) in a similar model in which TSST-1 was used.

Toxin-containing culture fluids of strain D4508 were prepared as follows. The organism was cultured at 37°C with high aeration in beef heart medium until the stationary phase. Cells were removed by centrifugation (10,000 × g, 30 min) and filtration (0.45-μm-pore-size filters; Millipore Corp., Bedford, Mass.). The toxin-containing culture fluids were dialyzed against distilled water for 2 days and lyophilized. The lyophilized preparations were subsequently solubilized with pyrogen-free distilled water, clarified by centrifugation (13,000 × g, 10 min), and adjusted to volumes which contained 100 μg of enterotoxin A per 0.1 ml, as estimated by serial Ouchterlony immunodiffusion (26). Finally, the concentrated culture fluids were diluted twofold by the addition of either normal rabbit serum or hyperimmune anti-enterotoxin A serum.

**RESULTS**

Strain D4508 of *S. aureus* was examined for the production of exotoxins belonging to the larger family of pyrogenic toxins which includes TSST-1, enterotoxins A to E, and scarlet fever toxins A to C. The strain, obtained from a patient with nonmenstrual TSS, was shown by Ouchterlony immunodiffusion analysis of 100×-concentrated culture fluid to make enterotoxin A but not TSST-1 or other known enterotoxins. In addition, Southern hybridization analysis indicated that strain D4508 lacked the TSST-1 gene, *tst*. DNA from strain MN8, used as a positive control, hybridized with *tst*, whereas DNA from strains MN8ON and MNHO, used as negative controls, failed to hybridize with *tst*.

Enterotoxin A from strain D4508 was partially purified by ethanol precipitation and isoelectric focusing and, upon SDS-PAGE, showed a protein band with a molecular weight of approximately 25,000, comparable to the molecular weight of control FRI 722-derived enterotoxin A (Fig. 1, lanes a and b) and similar to the reported enterotoxin A molecular weight of 27,100 (3). Western blot analysis with anti-enterotoxin A serum raised against FRI 722-derived toxin (lanes c and d) or antiserum raised against partially purified D4508 protein (lanes e and f) confirmed the identity of the 25,000-molecular-weight protein as enterotoxin A. The extra band seen in lanes b and f was considered a contaminant, since antiserum raised against purified enterotoxin A failed to react with that protein.

During these and subsequent experiments it was noted that strain D4508 showed great variability in the amount of enterotoxin A produced, in some experiments making less than 0.02 μg of toxin per ml of culture fluid and in others making approximately 3.2 μg/ml. Tests were conducted to evaluate the effect of culture media (beef heart, Todd-Hewitt [Difco Laboratories, Detroit, Mich.], and brain heart infusion [Difco]), length of culturing (from 1 to 24 h), different precipitation methods, and variable production by individual colonies to identify the source of the variability; none of the tests provided an explanation. Variable results were not obtained in related studies with strain FRI 722.

To assess the capacity of purified enterotoxin A to induce TSS-like symptoms in a rabbit model, we administered 0 or 100 μg of toxin in miniosmotic pumps to rabbits. All three rabbits receiving 100 μg of enterotoxin A showed symptoms of high fever, hypotension, diarrhea, and weight loss, and all three animals succumbed in less than 3 days. Animals that did not receive toxin remained healthy over the 7-day test period.

Concentrated culture fluid from strain D4508, mixed with either normal rabbit serum or anti-enterotoxin A serum, were comparably administered in miniosmotic pumps to rabbits. Rabbits treated with culture fluid containing approximately 50 μg of enterotoxin A plus normal rabbit serum showed symptoms of fever, diarrhea, and weight loss, and four of six animals succumbed during the test period. In contrast, animals treated with culture fluid containing 50 μg of enterotoxin A plus anti-enterotoxin A serum showed evidence of fever but otherwise remained healthy.

In a previous report (29), it was observed that culture fluids of strain D4508 reacted with antibodies against TSST-1 but did not contain TSST-1, thus suggesting that a cross-reactive protein was present. Therefore, 100×-concentrated culture fluids were evaluated for reactivity with polyclonal and four different monoclonal antibodies against TSST-1 by Western blot analysis. In all instances control TSST-1 reacted but the culture fluid remained negative (data not shown).
DISCUSSION

Previous studies (15, 17, 28, 29) indicated that S. aureus D4508 does not make TSST-1 yet has the capacity to induce TSS. Our studies confirm this lack of TSST-1 production and, furthermore, show that the strain lacks the structural gene for TSST-1. In addition, the strain does not make enterotoxin B, C1, D, or E. In an earlier study (29), in an attempt to identify the causative toxin made by strain D4508, it was reported that the strain made a protein, designated TSST-2, cross-reactive with antisera raised against TSST-1. We have not been able to demonstrate such a cross-reactive protein by Western blot analysis with both polyclonal and four monoclonal antibodies against TSST-1. Likewise, we have not detected a tst-cross-hybridizing DNA fragment. Taken together, these data suggest that the protein previously identified as TSST-2 was detected by an antiserum reaction with a product unrelated to TSST-1 but contaminating the toxin preparation used for immunization.

Staphylococcal enterotoxin A shares many biological and biochemical properties with TSST-1, thus raising the possibility that TSST-2 is enterotoxin A. Both enterotoxin A and TSST-1 are pyrogenic, predispose the host to lethal endotoxin shock, nonspecifically induce T-lymphocyte proliferation, and have near neutral isoelectric points and similar molecular weights (3, 5, 6, 27, 31). In addition, enterotoxin A is made by many strains that make TSST-1 and is highly lethal to rabbits (16, 24). Thus, unless great care is taken, enterotoxin A could contaminate TSST-1 preparations. In a report by Scott et al. (28), however, it was suggested that TSST-2 is not enterotoxin A but that enterotoxin A may have contributed to the pathogenesis of strain D4508. Our studies strongly suggest that enterotoxin A is, indeed, the major TSS-associated toxin made by that strain and that TSST-2 is not a required factor. Until the identity of the toxin in question is more clearly established and its role in TSS is clarified, the use of TSST-2 as a toxin designation does not seem warranted.

More recently, Hall et al. (17) have demonstrated the presence of another toxin made by strain D4508, and this factor also does not appear to be enterotoxin A, on the basis of its lower molecular weight and antigenic differences. As with TSST-2, this factor does not appear to be required for the strain to cause TSS symptoms.

In this study, we have noted that the amount of enterotoxin A made by strain D4508 is quite variable and have been unable to identify the source of the variability. It is proposed that variability in an as-yet unidentified nutrient in the culture media tested explains the variation in toxin amounts obtained. It is possible that this variable toxin expression explains the difficulty others have had in identifying TSS-inducing factors made by strain D4508.

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LITERATURE CITED


