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Analysis of Toxic Shock Syndrome Isolates Producing Staphylococcal Enterotoxins B and C1 with Use of Southern Hybridization and Immunologic Assays

Gregory A. Bohach,* Barry N. Kreiswirth, Richard P. Novick, and Patrick M. Schlievert

From the Department of Microbiology, Medical School, University of Minnesota, Minneapolis, Minnesota; and the Public Health Research Institute of the City of New York

A study was undertaken to evaluate the production of enterotoxin B (Ent B), EntC1, and toxic shock syndrome toxin 1 (TSST-1) by isolates of *Staphylococcus aureus* from patients with toxic shock syndrome (TSS) and from a variety of other sources. Levels of toxin in culture supernatants were measured by a quantitative immunodiffusion assay. Most vaginal TSS isolates produced TSST-1, either alone or with EntC1. However, strains that produced EntB or EntC1 but did not express TSST-1 were commonly isolated from patients with nonmenstrual TSS; EntB was usually produced alone or—rarely—with EntC1. These results were confirmed by probing DNA from representative isolates with an internal probe of the EntC1 gene (*entC1*). Extensive sequence homology between *entC1* and *entB* enabled detection of both genes under conditions of high stringency. The genomic location of *entC1* in strains producing both EntC1 and TSST-1 varied little but was dependent on the mammalian host. In contrast, the genomic location of *entC1* or *entB* in strains producing EntC1 or EntB alone was variable. These results suggest that these genes are contained on mobile elements.

Toxic shock syndrome (TSS) is an illness preceded by colonization or infection with *Staphylococcus aureus* and is characterized by fever, hypotension or shock, erythematous rash, desquamation, and multiorgan involvement [1]. Although the illness is systemic, the organism usually remains localized, a finding which suggests that extracellular toxins are involved in pathogenesis.

As a result of the association between high absorbency tampons and occurrence of TSS, initial studies primarily involved vaginal isolates of *S. aureus* from menstruating women. In 1981 Schlievert et al. [2] and Bergdoll et al. [3] independently identified an extracellular toxin that was later designated *toxic shock syndrome toxin 1* (TSST-1) [4]. TSST-1 was produced by nearly 100% of *S. aureus* isolates from women with menstrual cases of TSS, and symptoms of TSS could be induced in rabbits by injecting them with the purified toxin [5].

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* Present address: Department of Bacteriology and Biochemistry, University of Idaho, Moscow, Idaho 83843.

Please address requests for reprints to Dr. P. M. Schlievert, Department of Microbiology, Medical School, Box 196 UMHC, University of Minnesota, Minneapolis, Minnesota 55455.

More recently, it was noted that many nonvaginal isolates of *S. aureus* from patients with TSS do not produce TSST-1 [6–8]. Instead, enterotoxin B (EntB) was the only toxin produced by at least 38% of isolates from patients with nonmenstrual TSS [8]. Two independent reports also implicated EntC1 as a cause of TSS [9, 10]. Recently, our screening of clinical isolates detected two additional strains from patients with nonmenstrual TSS that produced only EntC1. Clinical symptoms seen in patients with nonmenstrual TSS are identical to those of patients with menstrually associated illness, in spite of the different toxins associated with each.

TSST-1, staphylococcal enterotoxins, and group A streptococcal pyrogenic exotoxins (SPEs) A–C [11–13] belong to a family of related pyrogenic toxins. Although the enterotoxins are distinguished from other pyrogenic toxins by their ability to cause staphylococcal food poisoning [14], these toxins share numerous biologic properties that could enable them to induce similar clinical syndromes, including TSS. These include induction of lymphocyte mitogenicity, immunosuppression, pyrogenicity, and enhancement of lethal endotoxic shock [15–19]. This could explain both the ability of TSST-1, EntB, and EntC1 to cause TSS and the demonstration of a TSS-like illness caused by SPE-producing group A streptococci [20].

In this paper we report our results of recent studies

of the roles of several pyrogenic toxins in the pathogenesis of TSS. Isolates of *S. aureus* from a variety of sources, including patients with TSS, were evaluated for production of TSS-associated toxins (TSST-1, EntB, and EntC1) by means of an immunologic assay. The presence and heterogeneity of the genomic location of genes for enterotoxin production were evaluated with the use of an internal probe for *entC1*.

Materials and Methods

Bacterial strains. Strains of *S. aureus* used in this study are listed in table 1. Strains were obtained from patients during acute episodes of *S. aureus* infection or from healthy volunteers and from sheep or cattle with mastitis.

Toxin purification. EntB, EntC1, and TSST-1 were prepared as described previously [2] from *S. aureus* strains MNLO [8], MNDON [21], and MN8 [22], respectively. The organisms were grown to stationary phase with aeration in dialyzable beef-heart medium [2]. Cultures were precipitated with four volumes of ethanol. Precipitates were redissolved in distilled water. Each toxin was partially purified by preparative thin-layer isoelectric focusing [2] in a pH gradient of 3.5–10. Additional purification was achieved by refocusing in a narrow pH range that was determined by the isoelectric point of each toxin.

Table 1. Source and toxin production by isolates of *Staphylococcus aureus*.

Strain designation	Source*	Toxin concentration (μg/mL)†		
		EntC1	EntB	TSST-1
MNDON	TSS-M	10.4	ND	ND
MN12	TSS-NM	10.4	ND	ND
717T	Healthy human (throat)	10.4	10.4	ND
MN78	TSS-NM	ND	10.4	ND
MNHO	TSS-NM	ND	20.8	ND
MN22	TSS-NM	10.4	ND	10.4
MN31	TSS-NM	20.8	ND	20.8
MN75	TSS-NM	10.4	ND	10.4
RN3170	Bovine mastitis	0.1	ND	0.6
RN5610	Sheep mastitis	1.3	ND	2.6
RN5625	Sheep mastitis	10.4	ND	10.4
MN8	TSS-M	ND	ND	20.8

* TSS-M and TSS-NM = menstrual and nonmenstrual, respectively.

† Concentration is given relative to original culture fluid. The lower limit of detection of the assay was 0.1 μg/mL. ND = not detectable.

Antisera preparation. Hyperimmune antisera were prepared by subcutaneous immunization [13] of American-Dutch belted rabbits with EntC1, EntB, or TSST-1 emulsified in Freund's adjuvant.

Toxin assays. *S. aureus* cultures were grown to stationary phase in 20 mL of dialyzable beef-heart medium with aeration [2]. Ten-milliliter amounts of culture were treated with 40 mL of ethanol at 4°C to precipitate toxins. The precipitates were recovered by centrifugation, and toxins were redissolved in 0.1 to 0.5 mL of H_2O. Samples were tested for reactivity with rabbit antisera by Ouchterlony immunodiffusion [23].

Toxins were quantitated by diluting the concentrates serially and testing them by Ouchterlony immunodiffusion. The end point of the assay was designated as the highest twofold dilution that produced a detectable precipitin arc. The sensitivity of the assay was determined by reactivity of antisera with toxin standards of know concentrations. With the use of this method, the lower limit of toxin detectable in the original culture fluid was 0.1 μg/mL.

Preparation of entC1 gene probe. We previously described the cloning of *entC1* on a 7.1-kilobase (kb) DNA fragment (pMIN101) from the genome of *S. aureus* MNDON [21]. The gene was localized on a 1.5-kb *Hin*dIII-*Cla*I restriction fragment that was subcloned into pUC13 for construction of pMIN114 (figure 1). To produce an internal gene probe, 1 μg of *Hin*dIII- or *Bam*HI-linearized pMIN114 was digested (0–180 minutes) with 1 unit of exonuclease *Bal*31 (New England Biolabs, Beverly, Mass.) to remove flanking DNA. Aliquots were mixed with 40 m*M* EGTA to terminate the digestion, and the DNA was precipitated with ethanol. End repair of digested DNA fragments was accomplished with Klenow fragment (International Biotechnologies, New Haven, Conn.). Synthetic *Hin*dIII or *Bam*HI linkers (New England Biolabs) were added with T_4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Excess linkers were removed by digestion with the appropriate restriction enzyme and subsequent agarose gel electrophoresis. Plasmids were extracted from the agarose [24], re-ligated, and transformed into *Escherichia coli* JM83 [25] by the method of Kushner [26]. Transformants were checked for EntC1 production by Ouchterlony immunodiffusion [27].

DNA preparations. *S. aureus* cultures were grown until the midexponential growth phase in Todd-Hewitt broth (Difco, Detroit). Cells were

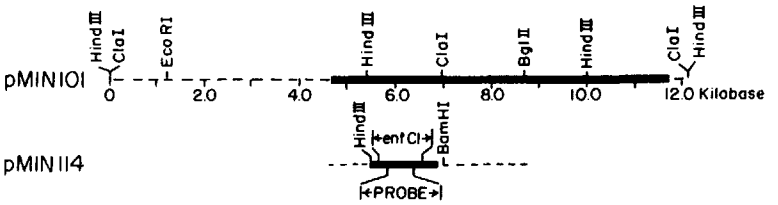

Figure 1. Production and characterization of *entCl* internal probe. Physical maps and important restriction sites of pMIN101 and subclone pMIN114 are shown. Heavy solid lines indicate cloned staphylococcal insert DNA, and dashed lines designate vector DNA. Boundaries of *entCl* and the internal probe produced by digestion of pMIN114 with exonuclease *Bal*31 are indicated. Analysis of nucleotide sequence homology between *entCl* and *entB* was derived from [31, 32]. Matched nucleic acids (:) and gaps (— — —) introduced for optimal alignment are shown. The portion of *entCl* used for probing is underlined.

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entB   1    ATGTATAAGAGATTATTTATTTCACATGTAATTTTGATATTCGCACTGATATTAGTTATTTCTACACCC   69
entCl  1    ATGAATAAGAGTCGATTTATTTCATGCGTAATTTTGATATTCGCACTTATACTAGTTCTTTTACACCC   69

       70   AACGTTTTAGCAGAGAGTCAACCAGATCCTAAACCAGATGAGTTGCACAAATCGAGTAAATTCACTGGTTTGATG  144
       70   AACGTATTAGCAGAGAGCCAACCAGACCCTACGCCAGATGAGTTGCACAAAGCGAGTAAATTCACTGGTTTGATG  144

      145   GAAAATATGAAAGTTTTGTATGATGATAATCATGTATCAGCAATAAACGTTAAATCTATAGATCAATTTCTATAC  219
      145   GAAAATATGAAAGTTTTATATGATGATCATTATGTATCAGCAACTAAAGTTAAGTCTGTAGATAAATTTTTGGCA  219

      220   TTTGACTTAATATATTCTATTAAGGACACTAAGTTAGGGAATTATGATAATGTTCGAGTCGAATTTAAAAACAAA  294
      220   CATGATTTAATTTATAACATTAGTGATAAAAAACTGAAAAATTATGACAAAGTGAAAACAGAGTTATTAAATGAA  294

      295   GATTTAGCTGATAAATACAAAGATAAATACGTAGATGTGTTTGGAGCTAATTATTATTATCAATGTTATTTTTCT  369
      295   GGTTTAGCAAAGAAGTACAAAGATGAAGTAGTTGATGTGTATGGATCAAATTACTATGTAAACTGCTATTTTTCA  369

      370   AAAAAAACGAATGATATTAATTCGCATCAAACTGACAAACGAAAAACTTGTATGTATGGTGGTGTAACTGAGCAT  444
      370   TGCAAAGATAATGTAGGTAAAGTTACAGGTGGC---------AAAACTTGTATGTATGGAGGAATAACAAAACAT  435

      445   AATGGAAACCAATTAGATAAATATAGAAGTATTACTGTTCGGGTATT------TGAAGATGGTAAAAATTTATTA  513
      436   GAAGGAAACCACTTGATAATGGGAACTTACAAAATGTACTTATAAGAGTTTATGAAAATAAAAGAAACACAATT  510

      514   TCTTTTGACGTACAAACTAATAAGAAAAAGGTGACTGCTCAAGAATTAGATTACCTAACTCGTCACTATTTGGTG  588
      510   TCTTTTGAAGTGCAAACTGATAAGAAAAAGTGTAACAGCTCAAGAACTAGACATAAAAGCTAGGAATTTTTAATT  585

      589   AAAAATAAAAAACTCTATGAATTTAACAACTCGCCTTATGAAACGGGATATATTAAATTTATAGAAAATGAG---  660
      586   AATAAAAAAAAATTTGTATGAGTTTAACAGTTCACCATATGAAACAGGATATATAAAATTTATTGAAAATAACGGC  660

      661   AATAGCTTTTGGTATGACATGATGCCTGCACCAGGAGATAAATTTGACCAATCTAAATATTTAATGATGTACAAT  735
      661   AATACTTTTTGGTATGATATGATGCCTGCACCAGGCGATAAGTTTGACCAATCTAAATATTTAATGATGTACAAC  735

      736   GACAATAAAATGGTTGATTCTAAAGATGTGAAGATTGAAGTTTATCTTACGACAAAGAAAAAGTGA  801  entB
      736   GACAATAAAACGGTTGATTCTAAAAGTGTGAAGATAGAAGTCCACCTTACAACAAAGAAATGGATAA  801  entCl
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pelleted; washed; resuspended in 50 m*M* Tris buffer, pH 7.8, containing 50 m*M* EDTA; and digested (1 hour, 37°C) with lysostaphin (500 μg/mL; Sigma Chemical Company, St. Louis). The cells were then lysed with SDS (0.56%) and treated (1 hour, 37°C) with 0.1% protease type XIV (Sigma); the resulting lysate was extracted with phenol and chloroform [28]. After ethanol precipitation, the nucleic acids were recovered by centrifugation and treated (1 hour, 37°C) with 0.2 units of pancreatic RNase (Sigma). The DNA was extracted again and stored precipitated at −20°C in ethanol.

Plasmid DNA was isolated from *E. coli* clones after cell lysis by treatment with lysozyme and boiling, as described by Holmes and Quigley [29]. Plasmids were partially purified by hydroxyapatite column chromatography and extraction with phenol and chloroform.

DNA hybridization. Probe DNA (1 μg) was radiolabeled with 130 μCi of [^{35}S]deoxyadenosine 5′-(α-thio)triphosphate ([^{35}S]dATP; NEN Research Products, Boston) with the use of a commercial nick-translation kit (Bethesda Research Laboratories, Gaithersburg, Md.). Radiolabeled DNA was separated from unincorporated [^{35}S]dATP by ethanol precipitation and redissolved in 100 μL of H_2O. The probe was denatured by diluting in 150 μL of I *N* NaOH and boiling for 5 minutes. After the probe was cooled on ice, it was neutralized with 150 μL of 1 *N* HCl and mixed with 1.5 mL of 0.5 *M* sodium phosphate buffer, pH 7.4.

Eight micrograms of DNA from *S. aureus* strains was digested with 20 units of *Cla*I. The restriction fragments were separated by electrophoresis in 0.75% agarose gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.) by the method of Southern [30]. In brief, the DNA was denatured by soaking the gels in 0.5 *M* NaOH–1.5 *M* NaCl and neutralized in 1.0 *M* Tris–1.5 *M* NaCl, pH 8.0. The gel was placed on a Plexiglas support

and overlayed with nitrocellulose. Transfer was accomplished overnight by capillary action in a bath of 10X SSC (3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0). The membrane was washed in 6X SSC, dried, and baked at 80°C for 2 hours.

Prior to hybridization the nitrocellulose was soaked (65°C, 3 hours) in 20 mL of prehybridization solution (5X SSC, 0.5% SDS, 0.1% Ficol 400, 0.1% polyvinylpyrolidone, 0.01% bovine serum albumin, 800 ng of denatured salmon sperm DNA). After prehybridization, the membrane was incubated (16 hours, 65°C) in 20 mL of hybridization solution (5X SSC, 0.5% SDS, 0.1% Ficol 400, 0.1% polyvinylpyrolidone, 0.01% bovine serum albumin, 10% dextran sulfate) containing labeled probe (2×10^6 cpm). The membrane was then washed in 2X SSC–0.5% SDS and dried. Bound probe was visualized by autoradiography on X-ray film (Eastman Kodak, Rochester, N.Y.) after overnight exposure.

Results

Patterns of toxin production. Routine screening of TSS isolates submitted to our laboratory indicated that nearly all menstrual TSS isolates expressed TSST-1 (table 2). In contrast, only 67% of nonmenstrual TSS isolates produced the toxin. The TSST-1-negative isolates produced either EntB (26%) or EntC1 (7%). Of the TSST-1-positive strains tested, 11% also produced EntC1, whereas none expressed EntB. These results are similar to those of others [6–8]. We analyzed the EntB and EntC1 production of several representative strains with regard to protein and DNA. Several enterotoxigenic *S. aureus* isolates used in this study, including some evaluated in table 2, were grouped according to source and profile of toxins produced (table 1). Most EntC1-positive strains evaluated in this study, including all of the animal isolates, also produced TSST-1. In contrast, we did not isolate an EntB producer that was also TSST-1-positive. Three human isolates produced EntC1 only. Simultaneous production of EntB and EntC1 occurred but was uncommon and was not associated with human illness in our survey. The only two strains with this toxin pattern that we have identified were from healthy volunteers.

Characteristics of entC1 probe. We previously reported cloning of *entC1* on a 7.1-kb insert in pBR328 for construction of the recombinant plasmid, pMIN101 [21] (figure 1). The gene was localized on a 1.5-kb *Hin*dIII-*Cla*I restriction fragment.

Table 2. Toxin production by isolates from patients with toxic shock syndrome (TSS) or probable TSS.

Source of isolates (no. of isolates tested)	No. (%) of isolates positive for indicated toxin(s)*			
	TSST-1 alone	EntB alone	EntC1 alone	TSST-1 and EntC1
Menstrual (39)	37 (95)	0	0	2 (5)
Nonmenstrual (31)	15 (48)	8 (26)	2 (7)	6 (19)

NOTE. TSST-1 = toxic shock syndrome toxin 1; Ent = enterotoxin.

* As determined by Ouchterlony immunodiffusion.

When subcloned into pUC13, the recombinant plasmid—pMIN114—expressed toxin that was indistinguishable from *Staphylococcus*-derived EntC1 [21]. An internal probe for *entC1* was constructed with the use of pMIN114. The gene boundaries (figure 1) were first localized by deleting flanking DNA and then testing transformants that received the deletion derivatives for the ability to express toxin. The internal probe used had 123 base pairs (bp) deleted from the 5′ end and 148 bp deleted from the 3′ end of *entC1*. For the resulting 530-bp internal region of *entC1*, 68% of the nucleotide sequence was homologous with the analogous region of *entB* (figure 1) [31, 32], and thus under stringent hybridization conditions was expected to cross-hybridize with *entB*.

Results of hybridization experiments. The *entC1* gene probe was nick translated and used to probe *Cla*I-digested *S. aureus* DNA (figure 2). Although 28 strains were analyzed, only representative results are shown. The degree of hybridization of the probe to *entC1* and *entB* was reflective of the extent of homology between the two genes. This was best demonstrated with the strain that was positive for both EntB and EntC1 (717T), in which both genes were detected. The probe did not hybridize to control DNA from strain MN8, which produces TSST-1 but not the enterotoxins. The size of the *Cla*I restriction fragment harboring *entB* or *entC1* was highly variable in TSST-1-negative strains and ranged from 3.5 kb to 5.8 kb for *entC1* and 2.6 kb to 7.2 kb for *entB*. Figure 2 also shows results of hybridization experiments for strains positive for both EntC1 and TSST-1 from sheep, cattle, and humans. None of these strains produced EntB that was detectable by immunologic assays, and correspondingly, *entB* was not indicated by hybridization results. In these EntB-negative strains, the genomic location of *entC1* was less vari-

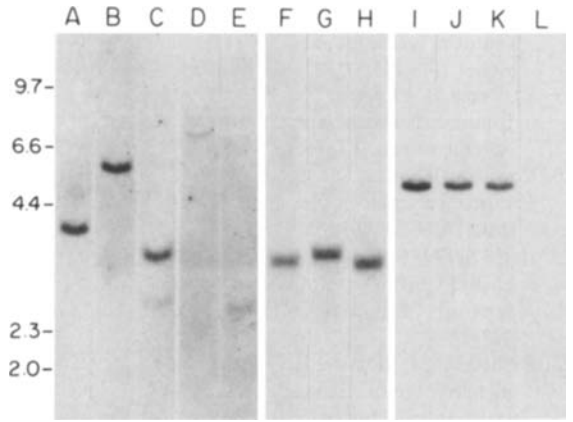

Figure 2. Southern blot of *Cla*I-digested DNA from representative isolates of *Staphylococcus aureus*. *Lane A*: MNDON; *B*: MN12; *C*: 717T; *D*: MN78; *E*: MNHO; *F*: MN22; *G*: MN31; *H*: MN75; *I*: RN3170; *J*: RN5610; *K*: RN5625; and *L*: MN8.

able than in the TSST-1-negative strains, although human and animal strains were clearly different. Sheep and bovine strains contained *entC1* on an invariable 5.0-kb *Cla*I restriction fragment (figure 2). In contrast, human strains showed slight variability; in these isolates, the restriction fragment harboring *entC1* was either 3.4 kb or 3.6 kb.

Discussion

Although TSST-1 was the first toxin shown to be associated with TSS, it was later recognized—through studies of nonmenstrually associated isolates—that EntB could also be responsible for the same illness [8]. In addition, two independent reports provided evidence that production of EntC1 may also be associated with nonmenstrual TSS [9, 10]. Recently, our laboratory detected two additional isolates (MNDON and MN12) from patients with TSS that produced only EntC1. Presently, we have no evidence that any other enterotoxin alone is associated with TSS. Although strains that produce TSST-1, EntB, or EntC1 often produce other enterotoxins, the other toxins generally are not produced alone.

In the present study we examined production of TSS-associated toxins (TSST-1, EntB, and EntC1) by representative isolates of *S. aureus*. We initially observed that EntC1 and TSST-1 were often coproduced whereas other toxin combinations were rarely or never produced. The genetic basis for these observations was examined by probing *Cla*I-digested DNA from representative strains with *entC1*. Because of the homology in their nucleotide sequences, *entC1* and *entB* were detected with the same gene probe. The correlation between the results of gene probing and those of immunologic assays for detection of Ent production was 100%.

Restriction fragments containing the enterotoxin genes were variable in size. The heterogeneity of sequences in flanking DNA might be attributed to the harboring of genes on mobile elements that are capable of insertion into the genome at variable positions. This possibility is consistent with observations of investigators who have provided evidence for both plasmid and chromosomal locations of *entC1* and *entB* [33–35] as well as for integration of enterotoxin plasmids into the staphylococcal chromosome [33].

All EntC1-positive, TSST-1-negative isolates contained *entC1* on *Cla*I restriction fragments of different sizes. However, human strains that coproduced EntC1 and TSST-1 contained the gene on either a 3.4-kb or a 3.6-kb fragment. Although isolates from sheep or cattle with this same phenotype had a nonvariable locus for *entC1*, the site was different from that of human strains. Thus, it appears that the presence of the TSST-1 gene, *tst*, influences the site at which *entC1* is located; some of the sites that *entC1* can occupy may be inaccessible to *entC1* when *tst* is present. Alternatively, the host may select a strain with a particular genetic makeup.

Fragments containing *entB* in the other five strains tested were heterogeneous in size (2.6–7.2 kb). In contrast, Ranelli et al. [36] have reported that *entB* is located on an invariable 5.5-kb *Cla*I restriction fragment. Our results suggest—as have the results of others [37]—that *entB* is located on a mobile element. Unlike *entC1* and *tst*, which frequently coexist, *entB* and *tst* appear to be mutually exclusive. Also, isolates coproducing EntB and EntC1 were found only rarely. Thus, both *tst* and *entC1* may in some ways interfere with integration of *entB* into the genome of *S. aureus*.

We are currently evaluating additional *S. aureus* isolates for toxin-production profiles and for variability in the location of genes for enterotoxin production. We hope to characterize the mechanisms involved in the mobility of *entB* and *entC1* and possibly of *tst*. Linkage of *entB* and *entC1* to large penicillinase-encoding plasmids has been reported [33]. However, we have been unable to demonstrate extrachromosomal enterotoxin genes in our studies. Our results are consistent with the location of *ent* genes on a phage or transposon. At least one staphy-

lococcal enterotoxin, EntA, is known to be encoded by a transmissable phage [38].

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Discussion

DR. JOHN ARBUTHNOTT. Dr. Bohach has demonstrated that it is not just a case of whether the organism has the genetic information to produce these factors, but it may be a question of where that information is located within the genome and what its surrounding genetic milieu is like.

DR. JAMES TODD. Dr. Bohach, you have mentioned that TSST-1 has been proven to be the cause of toxic shock in those patients infected with those strains. I suggest that this is the issue we are debating in this symposium, and we cannot necessarily accept that conclusion, at this stage. Second, you classified TSST-1 and the enterotoxins as pyrogenic exotoxins on the basis of four phenotypic criteria. What evidence do you have that those criteria define a truly unique biologic substance, and, specifically, what evidence do you have that there is genetic relatedness between those different toxins that you classified as pyrogenic exotoxins?

DR. GREGORY BOHACH. First, the fact that they are from the same organism and produce the same biologic properties provides circumstantial evidence, at least, that they might be involved in the same disease. Second, the genetic evidence implies that they are related. The structural genes for enterotoxin C1, enterotoxin B, and streptococcal pyrogenic exotoxin A have all been cloned and sequenced, and our analysis shows that they present significant nucleotide sequence homology. Enterotoxins B and C1 share sequence homology with a toxin of similar biologic properties from a completely different organism, pyrogenic exotoxin A.

DR. TODD. Can you give us an idea of the amount of homology between the streptococcal toxins and the enterotoxins?

DR. BOHACH. There is about 74% sequence homology between the two *ent* genes that we have looked at and about 58% homology between *speA*, which is a group A streptococcal toxin gene, and the enterotoxin C1 gene. These homologies are both highly significant.

DR. BENJAMIN SCHWARTZ. You presented numbers for the nonmenstrual patients who had staphylococcal enterotoxins B and C. You did not present any background rate of colonization with those toxin-producing *S. aureus* in the general population. It is necessary to show an association. Is it possible that an individual could be coinfected with two different *S. aureus* strains—one that produces exotoxins B and C and the other that may actually be responsible for the disease, being TSST-1-positive? In menstrual TSS, we know where to take the culture sample, but in nonmenstrual TSS the site of infection may not be apparent. I question whether you have detected the *S. aureus* that is responsible for the syndrome. My other question is, you have both definite and probable cases included in your presentation, and I wonder if the clinical manifestations are the same in the TSST-1-positive and the enterotoxin B and C cases, or whether the two different types of infection have different clinical spectra.

DR. BOHACH. It is true that in menstrual cases there is a chance that we may have picked up a strain that is not associated with the TSS that we observed, but in those strains we and others have shown that TSST-1 is the toxin involved. What we were trying to present here is that other toxins, including the two enterotoxins we looked at, may be involved, especially in nonmenstrual cases. In these cases, I think it is much less likely that we would have picked up an insignificant strain because these were usually isolated from sources such as blood cultures or wound infection.

DR. SCHWARTZ. Was there any clinical difference between the TSST-1-positive and the other cases?

DR. BOHACH. Only that what we have we tested is a wide variety of strains from various clinical situations, and the reason that many of the strains we tested were labeled as "probable" TSS-causing agents is that one of the criteria for TSS was missing. As was pointed out in this session, some of these criteria might not necessarily be seen in many cases.

DR. PATRICK SCHLIEVERT. We have published the numbers for enterotoxin B. For enterotoxin C we have not. The number of TSS isolates that are producing enterotoxin C alone is relatively small.

DR. BOHACH. We are currently screening hundreds of *S. aureus* strains from sites throughout the body from various staphylococcal infections, and the occurrence of enterotoxin C1/TSST-1 strains is less than 1%.

DR. EDWARD KASS. One of the great mysteries is the high frequency of antibody to a large number of the toxins without any overt disease. Yet after overt disease antibody does not necessarily follow. This

suggests that there may be either a great deal of low-level exposure to the toxins or some other source in nature. Having gotten this far in your identification of the amino acid sequence, have you looked at the catalogues to see if there are homologies with other known proteins elsewhere in nature that might give some hint of some other source, much as we see emerging with some of the gram-negative rods?

DR. BOHACH. We have done a computer search with the two enterotoxins that have been sequenced and also with the streptococcal A toxin, and, other than the fact that these three proteins possess significant homology among themselves, we could not find any significant homology with any other protein that was in the computer library.