Molecular Analysis of Pyrogenic Exotoxins from *Streptococcus pyogenes* Isolates Associated with Toxic Shock-Like Syndrome

ALAN R. HAUSER,† DENNIS L. STEVENS,‡ EDWARD L. KAPLAN,§ AND PATRICK M. SCHLIEVERT

Department of Microbiology† and Department of Pediatrics, World Health Organization Collaborating Center for Reference and Research on Streptococci,‡ University of Minnesota, Minneapolis, Minnesota 55455, and Division of Infectious Disease, Veterans Affairs Medical Center, Boise, Idaho 83702, and Department of Medicine, University of Washington, Seattle, Washington 98195

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Toxic shock-like syndrome (TSLS) is characterized by hypotension or shock, fever, multiorgan system involvement, and a concurrent group A streptococcal infection. We analyzed 34 streptococcal strains isolated from patients with clinically well-documented TSLS for their pyrogenic toxin profiles and M-protein types. Although strains of nine different M types were represented in the sample, 74% of the isolates were of either M type 1 or 3. It was determined that 53% produced streptococcal pyrogenic exotoxin type A under in vitro growth conditions and that 85% contained the gene encoding this toxin. These values are in contrast to the published value of 15% for the incidence of this gene in a sample of general group A streptococcal isolates. As has been found with all group A streptococci examined to date, regardless of disease association, 100% of TSLS-associated isolates contained the gene encoding pyrogenic exotoxin type B. This toxin was detectably produced by 59% of isolates. The gene encoding pyrogenic toxin type C was found in only 21% of isolates. We conclude that the pyrogenic exotoxin type A gene is associated with group A streptococcal strains isolated from patients with TSLS and may play a causative role in this illness. However, other factors are also likely to be important, since not all strains from patients with TSLS contained the A toxin gene.

In recent years numerous cases of a toxic shock-like syndrome (TSLS) caused by *Streptococcus pyogenes* have been reported (1, 6, 13, 30). Complications characteristic of the syndrome include hypotension or shock, fever, renal impairment, hypoalbuminemia, hypocalcemia, respiratory failure, vomiting and diarrhea, rash, and a concurrent group A streptococcal infection (30). The illness progresses rapidly and is usually quite severe, as indicated by the 30% mortality rate observed by Stevens et al. (30).

The multiorgan involvement typical of this illness even in the absence of bacteremia indicates that a toxin(s) may be involved in its pathogenesis. Indeed, the similarities between the complications given above and those seen in staphylococcal toxic shock syndrome (TSS) implicate a pyrogenic toxin.

Pyrogenic toxins are a group of acid-stable single-chain proteins ranging in molecular weight from 20,000 to 40,000. This group includes the staphylococcal enterotoxin serotypes A to E (SEA to SEE), the staphylococcal pyrogenic exotoxins A and B, toxic shock syndrome toxin type 1 (TSST-1), and the streptococcal pyrogenic exotoxins types A to C (SPE A to SPE C). These toxins are characterized by their similar biological activities: they induce fever, are nonspecific T-cell mitogens, enhance host susceptibility to endotoxin, suppress B-lymphocyte function, and cause a scarlet fever-like rash (3, 29, 33). Some members of the group, such as SPE A, SEB, and SEC, share significant sequence similarity (15). At least four of the staphylococcal pyrogenic toxins have been shown to play a significant role in the pathogenesis of staphylococcal TSS (21, 23, 24), whereas the SPEs, formerly called the scarlet fever toxins, have been shown to be important in the development of scarlet fever (7, 20).

The reason for the recent appearance of streptococcal TSLS has been the subject of much conjecture. Stevens et al. (30) have proposed that the syndrome is caused by streptococci elaborating SPE A and that the current outbreaks are due to the reemergence of strains capable of producing this toxin. However, TSLS-associated strains producing SPE B, SPE C, or both but not SPE A have been reported (4, 6, 30). It is also possible that the syndrome results from changing host susceptibility and immunity, selective antibiotic pressures, or a streptococcal antigen other than the SPEs.

This study was undertaken to examine a collection of TSLS-associated group A streptococcal isolates for their M-protein types, their toxin production profiles, and the presence of genes encoding the individual SPEs. The correlation between these characteristics and the M-protein serotypes of the strains was also investigated.

**MATERIALS AND METHODS**

**Bacterial isolates.** Thirty-four *S. pyogenes* strains reportedly isolated from patients with TSLS were submitted to our laboratories by physicians from 1986 through 1990. The patients were from diverse locations within the United States. For this study, the following working definition of TSLS was used: hypotension or shock, fever, and multiorgan system involvement concurrent with a group A streptococcal infection. The treating physicians who submitted the
strains used in this study were contacted to verify that each patient fulfilled the criteria for TSS. For those cases in which patient outcome data were available, a 37% mortality rate was observed. Patients presented in a variety of ways, including cellulitis, wound infection, or a flulike syndrome.

M-protein analysis. The M protein and/or opacity factor type and T type of each isolate was determined by the standard procedures used by the World Health Organization Collaborating Center for Reference and Research on Streptococci (University of Minnesota) (10, 14, 19, 32).

Ouchterlony immunodiffusion assays. Streptococci were grown to the stationary phase in 50 ml of dialyzable beef heart medium at 37°C in the presence of 7% carbon dioxide. When indicated, isolates were also grown in either 50 ml of dialyzable beef heart medium supplemented with 5% fetal bovine serum or additional glucose and glutamine or in 50 ml of Todd-Hewitt (Difco, Detroit, Mich.) medium. The broth was then treated with 200 ml of ethanol to precipitate toxins. Precipitates were dried and resuspended in 0.5 ml of distilled water, and insoluble material was removed by centrifugation in a microfuge for 10 min. At this stage, some supernatants were digested with hyaluronidase (600 U for 2 h at 37°C; Sigma Chemical Co., St. Louis, Mo.). The supernatants (20 μl) were tested for their reactivities with specific toxin antisera from rabbits by Ouchterlony immunodiffusion (22). The lower limit of toxin detectable by this method was 0.06 μg/ml of the original culture fluid. The presence of large concentrations of hyaluronic acid, even after hyaluronidase pretreatment, prevented reliable detection of toxins by enzyme-linked immunosorbent assay and Western blot (immunoblot) analysis.

DNA preparation. S. pyogenes cultures were grown overnight at 37°C in 10 ml of Todd-Hewitt broth. Cells were pelleted, washed twice in 1 ml of cold 0.2 M sodium acetate (pH 6.0), and resuspended in 500 μl of 10× TE-glucose (100 mM Tris, 10 mM EDTA, 25% glucose [pH 7.4]). After a 1-h incubation at 37°C in the presence of 20 μg of mutanolysin (Sigma), the cells were pelleted and resuspended in lysis buffer (100 mM Tris, 50 mM EDTA, 0.2% sodium dodecyl sulfate [SDS] [pH 8.5]). The cells were incubated with 250 μg of proteinase K (Sigma) for 30 min at 65°C and extracted with phenol, phenol-chloroform, and chloroform, and nucleic acids were precipitated by the addition of sodium acetate and ethanol.

Toxin gene probes. Toxin gene probes were obtained as diagrammed in Fig. 1. An approximately 500-bp internal DNA probe specific for speA, the gene encoding SPE A, was obtained by digesting pUMN107 (formerly pJS107) (17) with BamHI and BglII. A probe specific for speB, the gene which encodes SPE B, was obtained as follows. pUMN720 (12) DNA was digested with KpnI and ClaI. The resulting 612-bp fragment was an internal portion of speB. Probe DNA used to detect specifically speC, the gene which encodes SPE C, was obtained as follows. pUMN521 (9) was digested with DdeI to obtain a 654-bp fragment which includes a large part of the speC structural gene and 34 bp 3' to the speC stop codon. speA, speB and speC have little similarity between one another; therefore, it was expected that these probes would not cross-hybridize. This was confirmed by hybridization studies with streptococcal chromosomal DNA containing all three pyrogenic toxin genes (data not shown).

All digested plasmids were electrophoresed in agarose gels, and the appropriately sized fragments were extracted (27). A commercial nick-translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used to label each probe with [α-32P]dATP. Separation of labeled DNA

FIG. 1. Construction of pyrogenic exotoxin gene probes. The speA probe was originally obtained by digesting a cloned copy of this gene with Bal31 followed by the ligation of BamHI linkers (17). speB and speC probes were constructed by digestion with the indicated restriction enzymes.

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TABLE 1. Number of TSLS-associated isolates which have the specified phenotypes and genotypes

<table>
<thead>
<tr>
<th>M type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T type&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. (%) of isolates</th>
<th>No. (%) of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Protein</td>
<td>Gene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Toxin A</td>
<td>Toxin B</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>15 (44)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2/8/25/1-19</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>10 (29)</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>NK&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>12</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>49</td>
<td>14</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>66</td>
<td>12</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>18</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>34</td>
<td>18 (53)</td>
</tr>
</tbody>
</table>

<sup>a</sup> M types 2, 4, 22, and 66 were identified by opacity factor inhibition assays.

<sup>b</sup> Exceptions were as follows: one M type 1 isolate was nontypeable for T antigen; two M type 3 isolates were T type 3/B; one M type 3 isolate was T type 3/13.

<sup>c</sup> NK, not known.

<sup>d</sup> NT, nontypeable. All three nontypeable isolates were opacity factor positive.

from unincorporated [α-32P]dATP was achieved by ethanol precipitation and repeated 70% ethanol washes. Probe DNA was then denatured prior to use by incubation at 37°C for 5 min in 0.1 M sodium hydroxide.

**DNA hybridizations.** Purified chromosomal DNA was digested with PstI, electrophoresed in 0.7% agarose gels, and transferred to Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.) (28). The Nytran membranes were incubated for 2 h at 42°C in prehybridization solution which consisted of 6× SSPE (SSPE is 0.18 M NaCl, 10 mM sodium phosphate [pH 7.7], 1 mM EDTA), 0.5% SDS, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 50 μg of denatured salmon sperm DNA per ml. The membrane was then incubated for 16 h at 42°C in hybridization solution (6× SSPE, 0.5% SDS, 50% formamide, 10% dextran sulfate 500, 50 μg of denatured salmon sperm DNA per ml) containing labeled probe. The membranes were washed once in 6× SSPE-0.1% SDS for 30 min at room temperature and once in 1× SSPE-0.5% SDS for 30 min at 37°C and dried. Bound probe was visualized by autoradiography.

**RESULTS**

**M-protein types.** The TSLS-associated strains were of the M-protein types shown in Table 1. The majority of the isolates (74%) were of the M type 1 and M type 3 phenotype.

**SPE A.** The Ouchterlony immunodiffusion reactivities of streptococcal isolates with SPE antisera were summarized in Table 1. A total of 10 of 10 M type 3 isolates reacted with antisera to SPE A, whereas only 3 of 15 M type 1 isolates reacted with this antisera. Some isolates of other M types also reacted with SPE A antisera. Overall, 18 of 34 (53%) TSLS-associated strains tested elaborated SPE A, as assayed by Ouchterlony immunodiffusion.

Hybridization results, summarized in Table 1, indicated that 13 of 15 (87%) M type 1 and 10 of 10 (100%) M type 3 isolates tested contained a DNA sequence which hybridized to the speA probe. In all, 29 of 34 (85%) TSLS isolates tested contained a speA-like sequence. In each of the M type 1 isolates, this sequence was located on a 14-kb PstI DNA fragment, whereas in each M type 3 isolate, the sequence was contained on an approximately 23-kb PstI DNA fragment (Fig. 2 and Table 2). Isolates of M types 2, 18, 49, and 66 as well as several strains which were not typeable also contained speA-like sequences, but usually on different-sized PstI DNA fragments than were observed for M type 1 and 3 isolates.

To investigate the possibility that some streptococcal isolates at one time contained speA but lost this gene during passing, in the viratro stability of the speA genotype was examined and found to be stable even after 20 passages.

**TABLE 2.** Toxin RFLPs of TSLS-associated group A streptococci

<table>
<thead>
<tr>
<th>M type</th>
<th>No. of isolates</th>
<th>speA</th>
<th>speB</th>
<th>speC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>14</td>
<td>23</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>23</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>14</td>
<td>23</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>6.7</td>
<td>6.0</td>
<td>4.3</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>23</td>
<td>4.3</td>
<td>3.3</td>
</tr>
<tr>
<td>22</td>
<td>1</td>
<td>6.0</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>1</td>
<td>14</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>1</td>
<td>5.5</td>
<td>23</td>
<td>5.9</td>
</tr>
<tr>
<td>NT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>14</td>
<td>6.0</td>
<td>4.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> NT, nontypeable.

FIG. 2. Hybridization of the speA-specific probe to streptococcal chromosomal DNA restriction digested with PstI. The M-protein types of the isolates are indicated at the top, and the sizes of the hybridized fragments are shown at the left.
length polymorphism (RFLP) was detected for nearly every M type examined in this study (Table 2).

SPE C. Because of the relatively weak immunogenicity of SPE C, we were unable to reliably detect SPE C by the Ouchterlony immunodiffusion assay.

Hybridization results for chromosomal DNA from selected isolates with the spec probe indicated that only 21% of tested strains contained a speC-hybridizing gene. Since no two of these isolates were of the same M type, it was not possible to determine whether the RFLPs of this gene remained uniform within isolates of a particular M type. The pattern, however, did differ between M types (Table 2).

**DISCUSSION**

The majority (74%) of TSLS-associated group A streptococcal isolates were of M-protein types 1 or 3. Interestingly, these are the same two M types which have been associated with many of the streptococci isolated from recent acute rheumatic fever outbreaks in the United States (18). Gaworzewska and Colman (8) have reported that, in England, group A streptococcal isolates of M types 6, 49, and 81 increased and then decreased in frequency and were replaced by M types 1, 3, and 28 in the latter half of the 1980s. A similar emergence of streptococci of these M types appears to have occurred in the United States (14a) and may explain, in part, the high frequency of TSLS-associated organisms with these M types. A significant number of isolates, however, were of M types other than 1 and 3, making it unlikely that any factor responsible for the pathogenesis of TSLS is uniquely associated with one or two particular serotypes.

Our experiments indicate that speA is associated with TSLS. Of 34 isolates tested, 29 (85%) were shown to contain DNA sequences which hybridized to an internal speA probe. In contrast, a recent study (34) indicated that only 15% of group A streptococcal strains isolated from patients with a variety of illnesses contain speA. SPE A is an attractive candidate for playing a role in the pathogenesis of TSLS, since it shares many of the biological properties of TSST-1 and SEB, the major causative toxins of staphylococcal TSS. SPE A shares approximately 50% sequence similarity with SEB (15). Also, speA is carried by a phage (16) and is thus easily disseminated to streptococci of a variety of serotypes.

Interestingly, the majority of M type 1 strains examined in this study elaborated very small amounts of or no SPE A, such that this toxin could not be reliably detected by Ouchterlony immunodiffusion when these strains were grown in vitro. SPE A production was correlated perfectly with the presence of a speA-hybridizing sequence in all other serotypes except a single M type 18 isolate. SPE A production may be down-regulated under in vitro growth conditions in M type 1 organisms. This would result in the underestimation of SPE A-producing organisms in previously published reports on TSLS, which relied on Ouchterlony immunodiffusion assays for toxin testing (4, 6, 30). Expression of SPE A in these isolates was not affected by growth in Todd-Hewitt medium or dialyzable beef heart medium with or without 5% fetal bovine serum, additional glucose, or glutamine; these last three supplements have, at times, been used to amplify toxin production. Thus, we were unable to rule out the possibility that this toxin was also not expressed in vivo from these strains. It may be that the toxin is expressed at normal levels but is bound by hyaluronic acid upon excretion and, therefore, is unable to diffuse into the agarose during Ouchterlony immunodiffusion assays (25).
However, treatment of bacterial broth precipitates with hyaluronidase had no effect on toxin assay results. Alternatively, it is possible that the M type 1 streptococci examined in this study elaborate a previously undescribed toxin which shares a limited number of epitopes with SPE A, accounting for its diminished antibody reactivity. Finally, the toxin may be either intrinsically unstable or degraded by a bacterial protease. The similarity of the toxin RFLPs of non-SPE A-producing M type 1 strains with those of M type 1 strains which produce this toxin suggests that these isolates may have the potential to produce SPE A. Further studies are necessary to fully elucidate the expression of speA in M type 1 streptococci. Even with the absence of detectable SPE A production by these strains, 53% of TLSS-associated isolates elaborated this toxin in vitro.

It has been postulated that the current outbreaks of TLSS are due to the resurgence of group A streptococcal strains which produce SPE A (6, 30). Our results support this hypothesis. Evidence indicates that SPE A-producing strains may have been prevalent prior to 1940 (11), a time associated with a more severe form of scarlet fever which often resulted in death (2, 31). Interestingly, this illness shared a number of clinical features with TLSS (6, 26). The emergence of a milder form of scarlet fever coincided with the disappearance of SPE A-producing streptococci (26). The recent outbreaks of TLSS may be due to a reemergence of SPE A-producing organisms. Cyclical emergence and disappearance of group A streptococcal strains have been seen, and M types 1 and 3 are becoming more common. These M types may be more closely associated with speA than with other serotypes of group A streptococci, perhaps because of the acquisition of a defective speA-containing phage within their genomes at an evolutionarily earlier time (17). Alternatively, perhaps viable speA-containing phages are currently disseminating and converting SPE A-negative streptococci to SPE A-producing streptococci. Finally, it is possible that a combination of the two explanations given above is correct and that some serotypes of group A streptococci are more susceptible to lysogenic conversion by the speA-containing phage. M types 1 and 3 strains may be such strains. Thus, the cycling of M-type streptococci results in the cycling of organisms which are susceptible to the speA-containing phage and allow its dissemination.

The results of this study are compatible with the hypothesis that SPE A plays a causative role in TLSS by showing that the two are associated. The reason for the association of SPE A with TLSS rather than SPE B or C may be due to the increased potency of this toxin relative to those of the other toxins (20). It is also possible, however, that speA is genetically linked to one or more other genes which are required to induce TLSS.

Five isolates did not have a gene which hybridized to the speA probe and yet were associated with serious toxic shock-like disease. Each of these strains produced at least one other pyrogenic toxin. Interestingly, less than 100% of tampon-associated staphylococcal TSS isolates produce TSST-1, yet it is clear that TSST-1 is important in the development of this illness. Because quantitative analysis of toxin production was not done in this study, it remains a possibility that SPE A-negative strains elaborate high levels of other toxins, which allows them to cause TLSS. Genetic stability studies indicate that it is unlikely that these strains originally contained speA and then lost it after in vitro passaging. It remains possible that the affected individuals were infected by more than one strain of streptococci or Staphylococcus aureus and that the causative organism was not isolated by the treating physician. Alternatively, TLSS may have multiple etiologies.

All isolates contained a single copy of speB, although only 59% of these strains elaborated SPE B. These findings are not unique to TLSS-associated organisms but appear to be characteristic of all group A streptococci (11a).

Only 21% of isolates tested contained DNA which hybridized to the speC probe. Because of the weak immunogenicity of this toxin, we were unable to detect its production using Ouchterlony immunodiffusion assays and were thus unable to determine whether SPE C production correlated with the presence of speC. Interestingly, one representative of each serotype tested contained this gene.

Comparison of toxin RFLPs as a function of M type indicates that, with one exception, each tested M type has a unique hybridization pattern. Although different isolates within a given M type may have or lack the speA or the speC genes, if these genes are present, they are always within a PstI fragment of a given size. Further study is necessary to generalize these findings, but it may be that nearly all streptococci of a particular M type are characterized by a signature toxin RFLP and that this pattern differs from those of streptococci of other M types. It is possible that toxin RFLPs could be used as an adjunct to M typing and DNA fingerprinting (5) in categorizing and determining the relatedness of streptococcal isolates.

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