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Development of Streptococcal Pyrogenic Exotoxin C Vaccine Toxoids That Are Protective in the Rabbit Model of Toxic Shock Syndrome

John K. McCormick, * Timothy J. Tripp, * Stephen B. Olmsted, ‡ Yury V. Matsuka, ‡ Pamala J. Gahr, * Douglas O. Ohlendorf, † and Patrick M. Schlievert * *

Streptococcal pyrogenic exotoxin C (SPE C) is a superantigen produced by many strains of *Streptococcus pyogenes* that (along with streptococcal pyrogenic exotoxin A) is highly associated with streptococcal toxic shock syndrome (STSS) and other invasive streptococcal diseases. Based on the three-dimensional structure of SPE C, solvent-exposed residues predicted to be important for binding to the TCR or the MHC class II molecule, or important for dimerization, were generated. Based on decreased mitogenic activity of various single-site mutants, the double-site mutant Y15A/N38D and the triple-site mutant Y15A/H35A/N38D were constructed and analyzed for superantigenicity, toxicity (lethality), immunogenicity, and the ability to protect against wild-type SPE C-induced STSS. The Y15A/N38D and Y15A/H35A/N38D mutants were nonmitogenic for rabbit splenocytes and human PBMCs and nonlethal in two rabbit models of STSS, yet both mutants were highly immunogenic. Animals vaccinated with the Y15A/N38D or Y15A/H35A/N38D toxoids were protected from challenge with wild-type SPE C. Collectively, these data indicate that the Y15A/N38D and Y15A/H35A/N38D mutants may be useful as toxoid vaccine candidates. *The Journal of Immunology*, 2000, 165: 2306–2312.

S treptococcal toxic shock syndrome (STSS) is a severe, multisystem illness characterized by the rapid onset of fever and hypotension that can be considered a subset of invasive streptococcal disease (1, 2). Since 1980, there has been a largely unexplained resurgence of severe invasive disease due to group A streptococci (*Streptococcus pyogenes*), including necrotizing fasciitis and myonecrosis with or without STSS (3–9).

Both group A streptococci and *Staphylococcus aureus* produce potent exotoxins that belong to the family of pyrogenic toxin superantigens (PTSAgs) (10). PTSAgs simultaneously bind to class II MHC molecules and to the Vβ chain of the TCR outside of the conventional peptide groove (11). Through this interaction, these potent toxins subvert the normal immune response by forming a TCR-superantigen-MHC ternary complex (12) that stimulates T cells bearing the appropriate Vβ region (13). Because there are a limited number of Vβs and various superantigens recognize a subset of these (e.g., SPE C binds primarily to Vβ2; (13)), as many as 20% of all T cells can be stimulated. This results in extensive T cell proliferation with the consequent massive release of cytokines (14–18) which are believed to cause the most severe features of the illness.

The streptococcal pyrogenic exotoxins (SPEs), also known as erythrogenic toxins or scarlet fever toxins, include the serologically distinct types A, B (cysteine protease), C, F (DNase), G, and H, as well as streptococcal superantigen (19) and streptococcal mitogenic exotoxin Z (SMEZ) (20). Except for SPE B and SPE F, all of the other characterized PTSAgs are believed to share a similar three-dimensional structure (21–26). SPEs have been implicated as causative agents of STSS (2) and are responsible for the fever, rash, and severe clinical manifestations associated with scarlet fever. In particular, SPE A and SPE C are highly associated with severe disease (27), although this association is not 100%. For example, in a study that evaluated 16 consecutive cases of severe invasive streptococcal disease, of 9 patients who developed STSS, 8 died. Of the 16 isolates, 15 made either SPE A or SPE C, and the 1 isolate that did not make either PTSAg came from a patient with borderline invasive disease (erysipelas) (4). Production of recently characterized exotoxins (28) similar to SPE A and SPE C may account for cases of STSS that are not associated with these two toxins. However, PTSAs are clearly not solely responsible for invasive streptococcal disease. Cysteine protease (29) is invariably present in all clinical isolates and has been shown to be necessary for disease in a mouse model of soft tissue infection (30), and other factors are likely to be involved.

Epidemiologically, SPE C is not as well characterized as SPE A, primarily due to the higher association of SPE A with invasive disease and the poor immunogenicity of SPE C. SPE C is believed to exist as a dimer although the MHC binding site normally seen with other PTSAsgs (31, 32) is thought to be contained within the dimer interface (13, 33). A second MHC binding site present on SPE C, this may represent a novel mechanism of PTSAg-mediated
T cell stimulation where dimerized toxin can cross link two TCR and two MHC class II molecules simultaneously (13). Based on mutational analysis of staphylococcal enterotoxin B (SEB) that indicated the N terminus was critical for T cell activation (35), Yamaoka et al. (36) randomly mutated 14 residues at the N terminus of SPE C in an attempt to localize residues important for activity. That study indicated that residues Tyr1, Ala16, and Tyr17 were likely involved in TCR recognition, although the TCR binding domain of SPE C has yet to be completely mapped.

STSS and other invasive streptococcal disease often occurs in patients with no underlying disease. Along these lines, various groups have shown that a lack of protective immunity to the SPEs may contribute to host susceptibility (37–39). For example, patients with invasive disease had lower titers of anti-M1 and anti-streptococcal superantigen-neutralizing Abs compared with healthy controls (39). In another report, analysis of 132 clinical isolates from invasive episodes of streptococcal disease over a 12-year period revealed that although the isolates showed great genetic diversity, the speC gene was strongly associated with STSS (38). Furthermore, this study demonstrated a complete lack of SPE A-neutralizing Abs in patients with STSS and a low level of SPE A-neutralizing Abs in patients with uncomplicated bacteraemia compared with uncomplicated erysipelas (38). These studies indicate that the lack of immunity to the PtsSags is a strong risk factor for disease and that vaccine toxoids of the SPEs may protect against invasive disease and STSS.

Our research goals include the development of vaccine toxoids such that the immune system can target the causative toxin. In this study, we utilized the three-dimensional structure of SPE C (33) compared with other PtsSag structures, to predict residues important for binding to the TCR or class II MHC, or important for dimer formation. Single residues were mutated and assayed for the loss of mitogenic activity, and amino acids important for biological activity were altered to design double- and triple-site mutants of SPE C. These double- and triple-site mutants were evaluated for pyrogenicity, superantigenicity, toxicity, immunogenicity, and their ability to protect against SPE C-induced STSS.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains used in this study included Escherichia coli XL1-blue (Stratagene, La Jolla, CA) and DH5α (Life Technologies, Gaithersburg, MD) as cloning strains, and E. coli BL21 (DE3) (Novagen, Madison, WI) was used for expression of recombinant proteins. S. pyogenes T15P was used for purification of wild-type SPE C in some experiments (40). The plasmids used in this study included pMIN521 (41) as template for the speC gene, and pET-28 (Novagen) was used as the cloning vector for expression constructs. Kanamycin and ampicillin were both used at 50 μg/ml for E. coli containing appropriate plasmids. For cloning experiments, E. coli was grown in Luria-Bertani broth (42). For expression of SPE C molecules, E. coli and S. pyogenes were grown in dialyzed bovine heart medium (43). Strains were stored at −70°C in their appropriate broth supplemented with 20% glycerol (v/v) or freeze dried.

Cloning and site-directed mutagenesis

To predict SPE C residues that were important for interaction with the TCR or with MHC class II molecules, the SPE C structure (33) was aligned with the structures of toxic shock syndrome toxin 1 (TSST-1) (23, 25), SEB (21), and SEC (26). Residues on SEC that are known to interact with TCR (44) or residues on TSST-1 and SEB that are known to interact with MHC class II (31, 32) were used to predict amino acids on SPE C at similar positions. Residues Asp52, Tyr1, and Tyr17 were predicted to be located close to residues that interact with the TCR and residues His58 and Asn34 were predicted to be close to residues that interact with MHC class II molecules. For the initial generation of single-site mutants, the previously cloned wild-type speC gene (pUM521) was used. The inverse PCR Quick Change mutagenesis procedure (Stratagene) was used to introduce specific codon changes to create the individual mutants D12A, Y15A, Y17A, H35A, and N38D. With this strategy, mutant oligonucleotide primers (Table I) were used to replicate both strands of the entire plasmid using the high fidelity Pfu polymerase, while incorporating the desired mutation. The original template DNA was removed by digestion with the restriction enzyme DpnI that digests only methylated DNA and the remaining PCR products were transformed into E. coli DH5α. Each gene was sequenced to confirm that the correct mutation was incorporated. The double mutant Y15A/N38D was also constructed in a similar manner. To allow for more efficient expression, the Y15A/N38D mutant was amplified by PCR using primers SPE C-forward (5′-CCCCAGGCTCATTGAAAGACATT TCAGAATG-3′) and SPE C-reverse (5′-CCCCGCATTCCTTTTCTTTCTATA AATGGAAAACCATTG-3′) and cloned into the NcoI and BamHI sites (underlined in primers) of pET-28 resulting in pLP637. The triple mutant Y15A/H35A/N38D was constructed from pLP637 to create pJKM79 using the inverse PCR method with forward and reverse H35A/N38D primers. Due to the close proximity of these two mutations, the primers used to add the H35A mutation to pLP637 contained both H35A and N38D mutations. The wild-type speC (45) gene was also amplified from plasmid pUM521 using primers SPE C-forward and SPE C-reverse and cloned into pET-28 for the mutants pJKM90. To allow for high level production of the recombinant SPE C molecules, pLP637, pJKM79, and pJKM80 were transformed into the expression host E. coli BL21 (DE3) (39).

Toxin purification

The Y15A/N38D and Y15A/H35A/N38D mutants were expressed from E. coli BL21 (DE3) containing the appropriate recombinant plasmid by growth in dialyzed bovine heart medium to OD600 0.5 and induced with 0.2 mM isopropyl β-D-thiogalactoside. Cells were grown for an additional 3–4 h, and recombinant toxin was precipitated and purified as previously described (43, 46). Briefly, culture supernatants were precipitated with 4 volumes of absolute ethanol. Precipitates were resolubilized in pyrogen-free water, and purification was achieved by successive preparative flatbed isoelectric focusing using pH gradients of 3.5–10 and 6–8. Wild-type SPE C was produced from S. pyogenes T15P (40) or from E. coli BL21 (DE3) containing pJKM80 as described above. Purified proteins were analyzed for purity on SDS-10% polyacrylamide gels. Toxins were dialyzed extensively in pyrogen-free water and stored lyophilized until used.

T cell proliferation assays

Isolated human PBMCs or rabbit spleen cells were cultured in RPMI medium supplemented with 2% FCS and 1-glutamine (2 mM) at 2 × 10^5 cells/well. Serial 10-fold dilutions of purified wild-type or mutant SPE C proteins were added to the wells in quadruplicate. Plates were incubated in 7% CO2 at 37°C for 72 h, at which time each well received 1 μCi/well [3H]thymidine (Amersham, Arlington Heights, IL). After another 18 h, cells were harvested onto fiberglass filters (Whatman, Maidstone, England), and [3H]thymidine incorporation was assessed. Background was considered as counts from cells not treated with toxin (17).

Miniosmotic pump model of toxic shock syndrome (TSS)

The miniosmotic pump model of TSS was used to assess lethality of the various mutant toxins (47, 48). Miniosmotic pumps (Alza Pharmaceuticals, Palo Alto, CA) were preloaded with 500 μg wild-type or mutant toxin in PBS (pH 7.2). These devices are designed to release a constant amount of toxin over a period of 7 days with mimetic exposure of the animal to toxin during an actual infection. American Dutch belted rabbits were anesthetized with ketamine and xylazine, and preloaded miniosmotic pumps were implanted s.c. via a small incision in the left flank and closed through

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**Table 1. Primers used for construction of various SPE C mutants by site-directed mutagenesis**

<table>
<thead>
<tr>
<th>Mutant Residues</th>
<th>Corresponding Primers&lt;sup&gt;a&lt;/sup&gt; (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D12A</td>
<td>AACAGAAAAGGCTTACCATGATCA</td>
</tr>
<tr>
<td>Y15A</td>
<td>AGTGTATTTACTCCGATCAGCACTA</td>
</tr>
<tr>
<td>Y17A</td>
<td>TTATTTATGCGACCCATATAACCTT</td>
</tr>
<tr>
<td>H35A</td>
<td>TTTTCAACGACACCATACGAAATTACCTAA</td>
</tr>
<tr>
<td>N38D</td>
<td>AACACACATACGGCATTGATACCTCA</td>
</tr>
<tr>
<td>H35A/N38D</td>
<td>TTTTCACGACACCATACGAAATTACCTAA</td>
</tr>
</tbody>
</table>

<sup>a</sup> Underlined bases are areas in the primer that have been mutated to elicit the corresponding amino acid mutation. A complementary primer for each primer listed here was also made to facilitate the mutagenesis method outlined in Materials and Methods.
primary intention. Animals were monitored for symptoms of TSS and mortality was recorded for 15 days.

Enhancement of endotoxin shock model of TSS

The PTSAgs also have the ability to sensitize rabbits to the lethal effects of endotoxin induced shock. To address this activity, American Dutch belted rabbits were pretreated with sublethal doses of wild-type or mutant SPE C (5 μg/kg body weight) administered i.v. by the marginal ear vein (43). After 4 h, animals were challenged i.v. with a sublethal dose (10 μg/kg) of endotoxin from Salmonella typhimurium. Animals were monitored for symptoms of TSS, and mortality was recorded for 2 days.

Immunogenicity of recombinant SPE C molecules

In 2 independent experiments, 10 healthy American Dutch belted rabbits were prebled to determine preimmune serum titers. In each experiment, five rabbits were immunized with 25 μg of either the Y15A/N38D or the Y15A/H35A/N38D SPE C mutant at 2-week intervals (total of 3 immunizations) in IFA. Rabbits were bled at wk 7, and convalescent anti-SPE C titers were determined by ELISA with use of 1 μg wild-type SPE C to coat each well. Also on wk 7, animals immunized with the Y15A/N38D mutant were challenged with wild-type SPE C using the endotoxin enhancement model of TSS, whereas animals immunized with the Y15A/H35A/N38D mutant were challenged with 500 μg wild-type SPE C using the miniosmotic pump model of TSS. As a control, three rabbits were also immunized similarly with wild-type SPE C and titers were determined.

Results

Using site-directed mutagenesis, various single-site mutant SPE C molecules were generated based on both the three-dimensional structure of SPE C (Fig. 1) and predictions of binding sites for the TCR and MHC class II molecules. Mitogenic activity of the single-site mutants was tested with use of crude extracts from E. coli added to rabbit splenocytes. Residues that had significantly reduced activities were used to generate the double- and triple-site toxoids. The individual mutations were not studied in detail except that crude extracts had reduced mitogenic activity compared with wild-type SPE C. Structurally, we predicted five solvent-exposed residues (Asp12, Tyr15, Tyr17, His35, and Asn38) to be important for recognition of the TCR or the MHC class II or for dimer formation. Preliminary studies revealed that D12A did not have reduced activity. The Y17A SPE C mutant did have decreased mitogenic activity; however, the single-site mutants Y15A, H35A, and N38D showed the greatest reductions. Based on these activities, the double (Y15A/N38D)- and triple-site (Y15A/H35A/N38D) mutants were generated and cloned into the high level expression vector pET-28. The wild-type SPE C gene was also cloned into this vector. After purification, ~5 mg per liter were routinely obtained from this system (Fig. 2).

In standard mitogenicity assays using rabbit splenocytes (Fig. 3) or human PBMCs (Fig. 4), the activity of both multiple residue toxoids was reduced to background levels compared with wild-type SPE C. Recombinant wild-type SPE C and wild-type SPE C obtained from S. pyogenes T18P behaved similarly in all assays (data not shown). Both toxoids were nonpyrogenic compared with wild-type SPE C (Table II), further indicating that this activity is linked to superantigenic activity. The lack of pyrogenic activity of both toxoids also indicated that there were no significant amounts of contaminating endotoxin in the preparations (this method has a lower detection limit of 5 ng/ml endotoxin). Of critical importance, rabbits challenged with either toxoid exhibited no signs of toxicity in the two rabbit models of TSS (Table III).

After demonstrating that the two toxoids lacked biological activity, both were used to immunize rabbits in separate experiments. Significant Ab titers were raised for both toxoids compared with nonimmunized controls (Table IV). Under the same conditions, immunization with wild-type SPE C resulted in titers similar to

![FIGURE 1. Ribbon diagram of the three-dimensional structure of SPE C. Organized structures such as β strands and α helices are represented. Domains A and B are indicated. α carbons of the mutated residues are represented as spheres.](http://www.jimmunol.org/)

![FIGURE 2. Purified recombinant SPE C molecules. Purification was achieved as described in Materials and Methods. A, 10% SDS-PAGE analysis of wild-type SPE C (lane 1), Y15A/N38D SPE C (lane 2), and Y15A/H35A/N38D SPE C (lane 3) stained with Coomassie brilliant blue. Ordinate shows molecular mass markers. B, Representative elution profile (of wild-type SPE C) trace run on reverse-phase HPLC as previously described (63).](http://www.jimmunol.org/)
immunization with the SPE C toxoids (Table IV). Animals immunized with the double-site mutant were challenged with wild-type SPE C in the endotoxin enhancement model of TSS. Immune animals were completely protected from this challenge, whereas all nonimmune animals succumbed (Table IV). Animals immunized with the triple-site mutant were challenged with wild-type SPE C in the miniosmotic model of TSS. Again, all immune animals were protected from this challenge whereas nonimmune animals succumbed (Table IV).

Discussion

The crystal structure of SPE C has been solved at 2.4 Å (33), revealing a structure similar to those of other PTSAgs (21–26). Most data suggest that superantigenicity and lethal activity are coupled, and based on this hypothesis, it was reasonable to believe that single-site mutants in SPE C that had reduced mitogenicity would also likely have other impaired biological properties (pyrogenicity and toxicity). The crystal structure of staphylococcal enterotoxin C2 (SEC2) and SEC3 complexed with the TCR has been determined (44), and Tyr15 is analogous to residue Asn 23 in SEC2 and SEC3, residues that are located on the N-terminal α helix and interact with the TCR (44). Residues have been mutated in the N-terminal region of SPE C to examine the role of superantigenicity in erythema and found that mutants at positions Tyr15, Ala16, and Tyr17 had reduced mitogenicity and that erythema was likely attributable to T cell-stimulatory activity (36). Based on structural comparisons, our current data, and the previous mutagenesis results (36), it was concluded that Tyr15 is a critical residue for interaction with the TCR.

Interestingly, many of the residues analogous to the MHC class II α-chain-binding residues of SEA and SEB are buried in the SPE C dimer interface (13, 33). Residues His35 and Asn38 from SPE C are analogous to residues in the OB fold in TSST-1 and SEB, which are known to interact with MHC class II (31, 32). On the basis of this structural data, we predicted residue Asn38 to be important for MHC interaction although the N38I mutant reported earlier did not show altered activity (36). Although it is unlikely that a single mutation would disrupt dimer formation completely, whether this loss of activity depends on dimer formation or MHC class II interaction is not yet known. It is not known what effect these mutations had to the overall structure of the SPE C toxoids, although stability and reactivity of these molecules to Ab did not appear to be altered. Furthermore, the mutated residues were predicted to be solvent exposed, and alanine substitution would be expected to remove the side chain without altering conformation of the main chain or imposing any extreme steric or electrostatic effects (50). In our previous structure studies of TSST-1 mutants, changing such surface-exposed residues to alanine did not affect the overall structure (51).

Table II. Pyrogenicity of wild-type and toxoid SPE C proteins

<table>
<thead>
<tr>
<th>Protein Tested</th>
<th>Proteins administered in miniosmotic pumps</th>
<th>Proteins administered i.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Increased Fever Response (Δ°C ± SEM)</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>ND</td>
<td>1.1 ± 0.18</td>
</tr>
<tr>
<td>Y15A/N38D</td>
<td>ND</td>
<td>0.3 ± 0.13</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.14 ± 0.25</td>
<td>0.5 ± 0.42</td>
</tr>
<tr>
<td>Y15A/H35A/N38D</td>
<td>−0.3 ± 0.22</td>
<td>−0.66 ± 0.39</td>
</tr>
</tbody>
</table>

* A total of 500 μg of each protein were administered in s.c. implanted miniosmotic pumps. Temperatures were recorded on days 0 and 2.
* Student’s t test.
* Rabbits were treated with 5 μg/kg SPE C or various mutant proteins suspended in PBS administered i.v. Temperatures were recorded at 0 and 4 h.
SEA and SEE (34) as well as staphylococcal enterotoxin D (25, 49) all appear to have a second, higher affinity MHC class II binding site that is zinc dependent. With SPE C, MHC class II binding is believed to occur through a zinc binding site similar to that seen in SEA (24, 33). Mutational analysis within this region in SPE C has not been done but would be important to assess this region for MHC class II binding.

The SPEs were formerly known as scarlet fever or erythrogenic toxins because they were thought to be the causative agents of the scarlet fever rash. It is now known that the SPEs have a far more complex role in the pathogenesis of streptococcal disease. Purified SPEs cause STSS in rabbits, and various epidemiological studies have indicated that the SPEs are significantly associated with STSS (38, 52, 53). Streptococcal M1 and M3 serotypes cause the majority of invasive disease, and most M1 and M3 types produce SPE A. It has been shown that a widely disseminated SPE A-producing M3 clone has caused invasive disease in Ohio (54), North Carolina (55), Japan (56), and Minnesota (57) during 1994 and 1995. Musser et al. (9) also demonstrated that this same clone producing M3 clone has caused invasive disease in Ohio (54), North Carolina (55), Japan (56), and Minnesota (57) during 1994 and 1995. Musser et al. (9) also demonstrated that this same clone caused frequent cases of invasive disease before 1993. Our data further show that wild-type SPE C purified from group A streptococci or recombinant SPE C from *E. coli* produced highly lethal activity in the rabbit model.

In a study of soft tissue infection, recombinant SPE A purified from *Staphylococcus aureus* was used to immunize rabbits followed by s.c. challenge with SPE A-positive M1 or M3 streptococci (58). Of the SPE A-immune animals, 16 of 19 survived whereas only 4 of 20 nonimmune animals survived. Surviving animals showed soft abscess formation with a high influx of PMNs whereas nonimmune rabbits lacked inflammation. These authors proposed that toxin neutralization prevented TNF-α release due to superantigenicity, which was believed to down-regulate chemotactic receptors on PMNs to inhibit infiltration (59). Indeed, we believe a major evolutionary role for the SPEs is to allow for colonization by preventing infiltration of PMNs.

Other researchers have used a SPE A knockout M1 streptococcal strain and have argued that SPE A is not an important virulence factor in streptococcal fasciitis or myonecrosis (60). Using the SPE A-deficient strain, virulence was not diminished in a mouse model of bacteremia and myositis. Unfortunately, the mouse is not a good in vivo model because mice are highly resistant to the lethal effects of the PTSAgs. Furthermore, the strain with the disrupted speA allele retained nearly identical mitogenic activity compared with the wild-type strain, indicating that this strain produces another uncharacterized PTSAg.

Except for speB and speF, the presence of PTSAgs in *Staphylococcus aureus* and group A streptococci are variable traits. Not all group A streptococci produce either SPE A or SPE C, but expression of at least one of these two toxins is highly associated with invasive disease (27). SPE A is normally more highly associated with invasive disease, although this is not always the case. A study in a Memphis children’s hospital in the 1980s indicated that in this geographical region, the presence of the speC gene was more highly associated than the speA gene in clinical isolates from patients with bacteremia (27). Other novel streptococcal PTSAgs have been characterized including SMEZ and SMEZ-2 as well as SPE G, SPE H, and an incomplete gene for SPE J (28). If these novel PTSAgs are expressed at sufficient quantities, they could produce the same consequences as SPE A or SPE C.

In summary, it appears that the increased incidence and severity of streptococcal disease are likely due to both host factors and the changes in group A streptococci. Cases of noninvasive disease such as pharyngitis may serve as a reservoir for the dissemination of virulent strains to susceptible people (61). To reduce disease, regional surveillance of throat cultures may serve as an alternative to alert the medical community about the potential for invasive group A streptococcal illness (62). However, a functional vaccine may significantly reduce or eliminate severe invasive streptococcal disease and could also show benefits to less severe disease states such as pharyngitis, impetigo, or rheumatic fever. In this view, we have constructed a pentamutant of SPE A that is protective when used as an immunogen in rabbits challenged with wild-type SPE A (64). Here we have described the construction of two toxoid mutants of the SPE C superantigen that are protective against challenge with wild-type SPE C in two rabbit models of TSS. Both mutants were inactive for other biological activities such as fever

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**Table III. Lethality of wild-type and mutant SPE C proteins as determined in the miniosmotic pump and endotoxin enhancement models**

<table>
<thead>
<tr>
<th>Protein Tested</th>
<th>Miniosmotic pump model*</th>
<th>Enhancement of endotoxin shock model†</th>
<th>p‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>3/3</td>
<td>5/5</td>
<td>0.004</td>
</tr>
<tr>
<td>Y15A/N38D</td>
<td>0/5</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>5/5</td>
<td>4/4</td>
<td>0.008</td>
</tr>
<tr>
<td>Y15A/H35A/N38D</td>
<td>0/5</td>
<td>0.004</td>
<td></td>
</tr>
</tbody>
</table>

* A total of 500 μg of each protein were administered in s.c. implanted miniosmotic pumps, and mortality was recorded over 15 days.
† Student’s t test.
‡ Rabbits were treated with 5 μg/kg SPE C or various mutant proteins suspended in PBS administered i.v. After 4 h, 10 μg/kg purified *Salmonella typhimurium* endotoxin suspended in PBS were given i.v., and mortality was recorded over 48 h.

**Table IV. Immunogenicity and protective ability of SPE C toxoid molecules when used as vaccines against challenge with wild-type SPE C**

<table>
<thead>
<tr>
<th>Immunizing Agent</th>
<th>Preimmune§</th>
<th>Immune§</th>
<th>No. Dead</th>
<th>p#</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20</td>
<td>20</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>Y15A/N38D</td>
<td>208</td>
<td>9,600</td>
<td>0/5</td>
<td>0.008</td>
</tr>
<tr>
<td>None</td>
<td>208</td>
<td>320</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>Y15A/H35A/N38D</td>
<td>144</td>
<td>11,264</td>
<td>0/5</td>
<td>0.008</td>
</tr>
<tr>
<td>Wild-type</td>
<td>20</td>
<td>7,253</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

§ Rabbits were bled prior to administration of the first immunization. The average anti-SPE C titer of five rabbits is reported.
# Fisher’s exact test.
induction and superantigenicity. Only five amino acids were initially targeted for mutagenesis, yet three of these mutations significantly altered superantigenic activity which demonstrated the capability of the initial structural predictions. The failure of the D12A mutation to show an effect on superantigenicity was not surprising in that although the various structurally characterized PTSAgs have common structures, they are not identical, and certainly these structural variations are responsible for differences in biologic activities. Collectively, these data indicate that these two toxoids may be useful for inclusion with other vaccine candidates to help protect against invasive infection, scarlet fever, and STSS by group A streptococci.

Acknowledgments

We thank Greg Vath (Department of Biochemistry, University of Minnesota) for generating the ribbon diagram of the SPE C structure, Timothy Leonard (Department of Microbiology, University of Minnesota) for assistance in the preparation of Fig. 2, and Dinesha Walek (MicroChemical Faculty, University of Minnesota) for performing HPLC purification.

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