INFECTION AND IMMUNITY, Mar. 1996, p. 861–869 Vol. 64, No. 3
0019-9567/96/$04.00+0

Analysis of the Superantigenic Activity of Mutant and Allelic Forms of Streptococcal Pyrogenic Exotoxin A

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Received 18 October 1995/Returned for modification 29 November 1995/Accepted 22 December 1995

Infections with *Streptococcus pyogenes* (group A streptococcus) can result in the recently described streptococcal toxic shock syndrome (STSS), which is characterized by rashes, hypotension, multiorgan failure, and a high mortality rate. *S. pyogenes* isolates associated with STSS usually produce streptococcal pyrogenic exotoxin A (SpeA), a bacterial superantigen capable of stimulating host immune cells. Most of the symptoms of STSS are believed to result from cytokine release by the stimulated cells. To better understand the pathogenesis of STSS, we began studies on the SpeA-immune cell interaction. We generated 20 mutant forms of SpeA1 (SpeA encoded by allele 1), and the mutant toxins were analyzed for mitogenic stimulation of human peripheral blood mononuclear cells, affinity for class II major histocompatibility complex molecules (DQ), and disulfide bond formation. Residues necessary for each of these functions were identified. There are four alleles of *speA*, and STSS strains usually contain either allele 2 or allele 3. The product of allele 2, SpeA2, had slightly higher affinity for the class II MHC molecule compared with SpeA1 but not significantly greater mitogenic activity. SpeA3, however, was significantly increased in mitogenic activity and affinity for class II MHC compared with SpeA1. Thus, we have evidence that the toxin encoded by some of the highly virulent *S. pyogenes* STSS-associated isolates is a more active form of SpeA.

Streptococcus pyogenes (group A streptococcus) is a gram-positive pathogen that can cause pharyngitis (strep throat) and scarlet fever, as well as various suppurative skin infections such as cellulitis, erysipelas, impetigo, and necrotizing fasciitis (7). *S. pyogenes* infections are associated with the development of the nonsuppurative sequelae of rheumatic fever and acute glomerulonephritis (8). A number of streptococcal surface components and secreted products, including M protein, hyaluronic acid capsule, hemolysins, various enzymes, and the pyrogenic exotoxins, are believed to play an important role in infection. There are three streptococcal pyrogenic exotoxins, SpeA, -B, and -C, two of which, SpeA and -C, are encoded on temperate bacteriophage (9, 40). SpeB is a chromosomally encoded cysteine proteinase found in almost all *S. pyogenes* isolates. The pyrogenic exotoxins are also known as erythrogenic toxins or scarlet fever toxins, as they are thought to be responsible for the rash of scarlet fever.

From the 1900s to the late 1980s, the number of severe *S. pyogenes* infections decreased greatly. This decrease was due in part, although not entirely, to the widespread use of antibiotics (29). In recent years, however, there was a resurgence of acute group A streptococcal infections in both the United States and Europe (18, 31, 32, 38). A large percentage of these infections were characterized by rashes of various types, hypotension, multiorgan failure, and a high mortality rate. These symptoms were reminiscent of those associated with toxic shock syndrome, caused by *Staphylococcus aureus*, and this similarity in clinical features led to the designation of streptococcal toxic shock-like syndrome (STSS) for these invasive streptococcal infections (4). There is a correlation between STSS and SpeA-producing streptococcal strains, and the majority of strains cultured from patients in the United States with STSS express SpeA. One investigation of clinical isolates from patients with STSS revealed that 85% of these patients harbored strains positive for production of SpeA (16). In comparison, studies by Yu and Ferretti indicated that only 45% of scarlet fever isolates and 15% of non-disease-associated isolates carry *speA* (42).

SpeA (molecular weight, 25,787) is a bacterial superantigen (3, 36). Superantigens are believed to clonally activate and expand T cells through interaction with the T-cell receptor (TCR) (28). This activation requires presentation by major histocompatibility complex (MHC) class II molecules, and each superantigen activates a specific subset of the total T-cell repertoire. SpeA preferentially stimulates human T cells bearing Vβ chains 2, 8, 12, 14, and 15 (1, 10, 27, 39), and thus SpeA, similar to other superantigens, is able to activate a much larger proportion of the T-cell repertoire than conventional peptide antigens. The stimulated immune cells release the bioactive cytokines interleukin-2, tumor necrosis factor alpha, and gamma interferon, and this cytokine release produces the fever, hypotension, and other symptoms associated with STSS (2, 9, 28). Similar symptoms result when purified SpeA is administered to laboratory animals (26).

Group A streptococcal strains encoding specific *speA* alleles have been implicated in STSS outbreaks (34). There are four naturally occurring *speA* alleles, and three of these, *speA1*, *speA2*, and *speA3*, encode toxins differing by a single amino acid. The toxin encoded by *speA4* is 9% divergent from the other three, with 26 amino acid changes. Strains expressing *speA2* or *speA3* have caused the majority of STSS episodes in the last decade. The association of STSS with isolates containing these alleles suggests that SpeA2 and SpeA3 may be more toxic forms of this pyrogenic exotoxin.

To further understand the pathogenesis of STSS, we began studies on SpeA. These initial studies were to characterize the ability of SpeA to interact with immunocytes, and we undertook a mutational analysis of SpeA to define regions of the toxin important for this activity. Here, 20 mutant forms of

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SpeA are analyzed for their mitogenic activities and their affinities for class II MHC molecules. To determine if there was any significance to the association between STSS outbreaks and *speA2* and *-3*, the superantigenic properties of the toxins encoded by *speA1*, *speA2*, and *speA3* were compared. Finally, the importance of a potential disulfide loop in the ability of the toxin to stimulate immunocytes and bind to class II MHC molecules was evaluated.

MATERIALS AND METHODS

Purification of SpeA from *S. pyogenes*. One-liter cultures of *S. pyogenes* Ros (generous gift of D. L. Stevens, Idaho VA Medical Center) were grown in NCTC-135 medium (Gibco/BRL, Grand Island, N.Y.) supplemented with glucose (21). Toxin was partially purified from cell-free culture filtrates by differential solubility in ethanol and acetate-buffered saline (24). Toxin which had been precipitated four times was redissolved in 0.1 M imidazole-acetic acid (pH 5.0) and applied to a QAE-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) jacketed column. The toxin was eluted as a single peak with a NaCl gradient as described previously (12). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of purified SpeA revealed a single band with the expected molecular mass of SpeA (25.8 kDa). The toxin was dialyzed against phosphate-buffered saline (PBS) and stored at −20°C.

Construction of pET15b-*speA1*. One hundred fifty nanograms of plasmid pA2 (41) containing the *speA* gene (kindly provided by J. J. Ferretti, Oklahoma City, Okla.) was used as a template to amplify a 663-bp DNA fragment by PCR using primers 19b-A1 (5′-CCCCATATGCAACAAGACCCCGAT-3′) and 19b-A2 (5′-GGGGGATCCTTACTTGGTTGTTAG-3′). These primers encode terminal *Bam*HI and *Nde*I restriction sites, respectively. After digestion with *Bam*HI and *Nde*I (Gibco/BRL), the DNA fragment, which encodes the mature protein without the leader peptide, was cloned into *Bam*HI- and *Nde*I-digested pET15b (Novagen, Madison, Wis.), producing the construct pET15b-*speA1*. The complete nucleotide sequence of the inserted fragment was confirmed by the dideoxy-chain termination method (35). In *Escherichia coli* BL21(DE3) (Novagen), this construct expresses a fusion protein consisting of an N-terminal six-histidine-residue tag and SpeA1.

Generation of point mutations in *speA*. Site-directed mutagenesis of *speA1* was performed by using PCR with oligonucleotides containing the desired nucleotide substitution (17). Briefly, 150 ng of pET15b-*speA1*, the mutant oligonucleotide, and either primer 19b-A1 or primer 19b-A2 were used to generate two *speA* fragments with complementary ends. A second PCR was performed with the two overlapping *speA* fragments and flanking primers 19b-A1 and 19b-A2 to generate the full-length mutated *speA* gene. This PCR product was then digested with *Bam*HI and *Nde*I and inserted into pET15b as described above. The complete nucleotide sequences of both strands of each mutated *speA* were determined by the dideoxy-chain termination method to ensure that only the single desired mutation was present.

Recombinant toxin nomenclature. Recombinant SpeA1 (rSpeA1) amino acid substitution mutants were named according to the original amino acid, its position in the mature toxin, and the resulting amino acid. For example, for rSpeA1-N20A, amino acid residue 20 was changed from asparagine to alanine. All mutant recombinant proteins generated contained single amino acid substitutions except for rSpeA1-S51L,N55A and rSpeA1-C87S,C90S, which have two substitutions. rSpeA1 is the toxin encoded by *speA1* (40). rSpeA2 (also referred to as rSpeA1-G80S) is the toxin encoded by *speA2*. The toxin encoded by *speA3*, SpeA3, is also termed rSpeA1-V76I.

Expression and purification of rSpeA. Expression and purification of the recombinant toxins by using the pET expression vector was as described by manufacturer (Novagen). In brief, *E. coli* BL21(DE3) was transformed with pET15-*speA* constructs for production of recombinant toxins. In this background, *speA* is under the control of a T7 promoter, and the T7 polymerase gene is on the *E. coli* chromosome under the control of an isopropylthio-D-galactopyranoside (IPTG)-inducible *lac* promoter. Cultures were grown to mid-exponential phase and induced to express toxin by the addition of 0.4 mM IPTG (Sigma Chemical Co., St. Louis, Mo.). Cultures were grown for an additional 3 h after induction, harvested by centrifugation, and disrupted by sonication. rSpeA preparations were purified by metal chelation chromatography using His-Bind resin (Novagen). One hundred to 500 μg of toxin was digested with 1 μg of thrombin (Novagen) for 16 h at room temperature. The toxin was then purified from the His-tag leader sequence by ultrafiltration with 10,000-molecular-weight cutoff filters (MSI, Westboro, Mass.). In *E. coli* BL21(DE3) (Novagen), this construct expresses a fusion protein consisting of an N-terminal six-histidine-residue tag and SpeA.

Generation of polyclonal antisera recognizing SpeA. Female New Zealand White rabbits were immunized subcutaneously with 50 μg of commercially available SpeA1 (Toxin Technologies, Sarasota, Fla.) in complete Freund's adjuvant (Gibco/BRL). Subsequent immunizations of 25 μg of toxin were administered at week 3 and then every 2 weeks in incomplete Freund's adjuvant (Gibco/BRL). Sera were first collected at week 6.

Western blot (immunoblot) analysis of rSpeA. Each of the mutant toxins and allelic forms was screened for instability by Western analysis. Toxins were analyzed by SDS-PAGE (12% acrylamide) and electroblotted to nitrocellulose. The nitrocellulose filters were incubated overnight in PBS supplemented with 5% low-fat dry milk and then stained with polyclonal rabbit antiserum against SpeA1. Anti-SpeA antibody binding was detected with horseradish peroxidase-labeled goat anti-rabbit antibody. Bands were visualized with 4-chloro-1-naphthol (Sigma).

SDS-PAGE analysis. To look for the presence of disulfide bond formation between cysteine residues of rSpeA1, 2-μg aliquots of purified toxins were mixed with gel running buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 0.1% bromophenol blue, 10% glycerol) with or without 2-mercaptoethanol (final concentration, 1%). The samples were then boiled for 5 min and electrophoresed for 5 h at 40 mA on an SDS–12% polyacrylamide gel. Protein bands were visualized by staining with Coomassie brilliant blue R250 (Bio-Rad, Melville, N.Y.).

Mitogenicity assays. Heparinized whole blood was obtained from healthy donors. Samples were fractionated on Ficoll-Paque (Pharmacia Biotech, Piscataway, N.J.), and the peripheral blood mononuclear cells (PBMCs) were harvested and washed three times in PBS. Then cells (10^5) were added to 96-well U-bottom plates in 200 μl of complete RPMI 1640 supplemented with 10% fetal calf serum (FCS). PBMCs were incubated for 72 h at 37°C with various concentrations of rSpeA toxins under atmospheric conditions of 5% CO_2; 1 μCi of [^{3}H]thymidine (ICN Biochemicals, Costa Mesa, Calif.) was added to each well, and the cells were incubated for an additional 24 h. Cells were harvested onto glass fiber filters, and [^{3}H]thymidine uptake was quantitated by liquid scintillation counting. For each mutant toxin, PBMCs from at least three distinct donors were used.

Flow cytometry of PBMCs. PBMCs (10^6) from healthy donors were incubated with toxins at a concentration of 1 μg/ml for 4 days. Cells were harvested, washed three times with PBS, and applied to a FACScan flow cytometer (Becton Dickinson).

Cell lines. L-cell transfectants L66 (vector only) and L54.1 (DQβ3/DQα2) were the generous gift of Robert Karr, Monsanto Company (25). Transfectants were maintained in suspension in petri dishes in Dulbecco modified Eagle medium (DMEM) with 10% FCS, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 250 μg of the neomycin analog G418 per ml for selection. Before use, transfectants were examined by fluorescence-activated cell sorting analysis with fluorescein isothiocyanate-labeled anti-HLA-DQ3 (KS13) to confirm the expression and surface localization of the DQ molecule. Antibody KS13 was the generous gift of Soldano Ferrone, New York Medical College, Valhalla, N.Y.

Radiolabeled rSpeA binding assays. rSpeA1 was iodinated by using chloramine-T (Sigma). One hundred micrograms of toxin was incubated with 0.5 mCi of $Na^{125}I$ and 5 μl (5 mg/ml) of chloramine-T in 100 μl of 100 mM Tris–150 mM NaCl (pH 7.4) for 10 min. The reaction was terminated by the addition of 20 μl (5 mg/ml) of sodium metabisulfate (Sigma). Labeled toxin was separated from unincorporated radioactivity on a 1-ml Sephadex G-25 column, which had been preequilibrated with PBS. The K_d of rSpeA-DQ interaction was determined by incubating 10^6 L54.1 cells (expressing class II MHC) with various concentrations of ^{125}I-rSpeA in a total volume of 100 μl of DMEM–10% FCS–0.1% sodium azide. Nonspecific binding was estimated by incubating separate tubes with unlabeled competitor toxin at a concentration 100 times greater than that of labeled toxin. Cells were incubated at 37°C for 4 h with agitation every 20 min and then pelleted through an oil gradient (80% dibutyl phthalate, 20% olive oil). Pellets were cut from the tubes, and cell-associated ^{125}I was measured on a gamma counter.

K_i determinations were evaluated in a similar fashion except that additional tubes containing various concentrations of ^{125}I-rSpeA plus unlabeled mutant competitor were analyzed. Lineweaver-Burk plots of the reciprocal of toxin bound versus toxin free were used to determine inhibition constants (37).

Structure of SpeA. Predicted ribbon structure of SpeA was generated by the Swiss Model Automated Protein Modelling Server, Glaxo Institute for Molecular Biology, Geneva, Switzerland. Primary amino acid sequence of SpeA was modeled on the crystal structures of staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin E (SEE). Crystal coordinates for SEA and SEE are from the Brookhaven Database Crystal Coordinates and were deposited by Swaminathan and Sax (38a). Structure is viewed by using the Raswin Molecular Graphics Viewer software, version 2.4, 1994 (R. Sayle, Department of Computer Science, University of Edinburgh, Edinburgh, United Kingdom).

RESULTS

rSpeA and SpeA produced by *S. pyogenes* have equivalent mitogenic activities. To facilitate mutagenesis of *speA* and purification of the toxins encoded by the mutant alleles, we inserted the *speA* gene into the *E. coli* expression vector pET15b (Novagen). Expression from the pET15b-*speA* construct re-

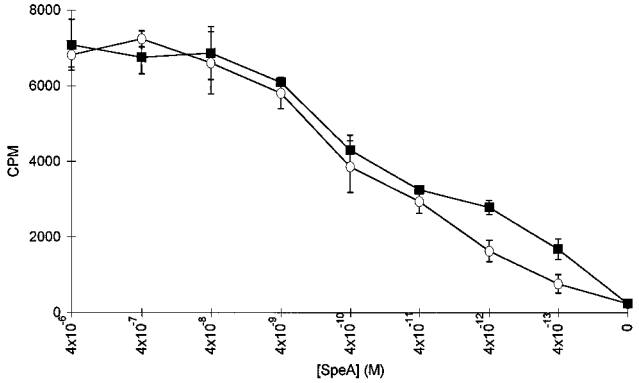

FIG. 1. Mitogenic activities of SpeA and rSpeA. SpeA was isolated from *S. pyogenes* Ros (SpeA3; ○), and rSpeA was isolated from the *E. coli* expression system (rSpeA3 [rSpeA1-V76I]; ■) as described in Materials and Methods. Human PBMCs were incubated with various concentrations of the toxins for 3 days, after which cells were pulsed with [^{3}H]thymidine for 16 h and harvested onto glass fiber filters, and incorporated counts were assessed by liquid scintillation.

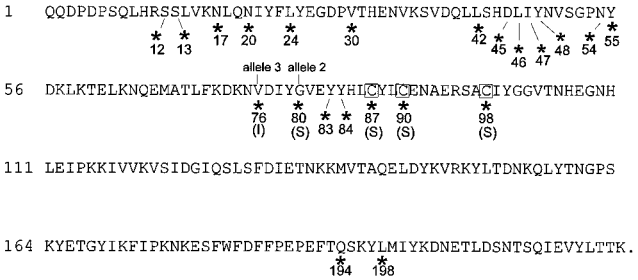

FIG. 2. Positions of SpeA1 amino acid substitutions. The primary amino acid sequence of SpeA1 is shown. Asterisks indicate positions of mutated residues; boxes highlight cysteine residues. Numbers below asterisks indicate numerical positions of residues in the primary amino acid sequence. Positions of allelic substitutions are as indicated. Except where noted in parentheses, all substituted residues were changed to alanine. Mutant toxins were generated as described in Materials and Methods.

sulted in the production of SpeA that lacked the native leader sequence but had a histidine-rich leader sequence (His-tag) encoded by the pET vector. The chimeric SpeA protein was purified from *E. coli* extracts by using a resin that selectively binds histidine residues as described in Materials and Methods. After elution from the column, the histidine-rich leader peptide was removed by thrombin cleavage. After cleavage with thrombin, four additional amino acids, glycine, serine, histidine, and methionine, remain on the amino terminus of rSpeA.

To confirm that this expression system was appropriate for these studies, the mitogenic activity of rSpeA was compared with the mitogenic activity of SpeA purified from a clinical *S. pyogenes* isolate. There are four known *speA* alleles. Toxin was purified by standard techniques from an *S. pyogenes* strain (Ros) that contains *speA3*. Therefore, for the comparison, SpeA was purified from *E. coli* containing a recombinant pET-*speA* construct that encoded *speA3*. The mitogenic activities of the wild-type and recombinant toxins were measured as a function of their abilities to stimulate human PBMCs. As shown in Fig. 1, there were no apparent differences in the stimulatory activities of the two toxin forms at the concentrations tested. A toxin preparation that had been heat denatured (100°C, 10 min) showed no activity, indicating there was no significant lipopolysaccharide contamination of the recombinant toxin preparation (data not shown). We concluded that the *E. coli* expression system would be suitable for production of SpeA for these studies.

Generation of rSpeA1 mutants. To identify amino acids of SpeA necessary for superantigenic activity, 22 site-directed mutations of *speA1* were generated as described in Materials and Methods. *speA1* refers to the original *speA1* gene isolated from bacteriophage T12 (40). Two of the mutations generated changed *speA1* to *speA2* and *speA3* (34). *speA2* encodes a toxin with a serine residue at position 80, while *speA1* encodes a glycine at that position. *speA3* encodes a toxin with an isoleucine at position 76, while *speA1* encodes a valine residue at that site.

The positions of the 20 additional amino acid substitutions generated in SpeA1 are shown in Fig. 2. Sixteen of the residues were chosen on the basis of their identity with residues known to be important for activity of the closely related bacterial superantigen staphylococcal enterotoxin B (SEB) (22). The wild-type amino acid of SpeA1 was changed to an alanine residue at each of these 16 positions. Three modifications were made to screen for residues forming a disulfide bond; these mutations resulted in the substitution of cysteines with serine residues at positions 87, 90, and 98. One modification was chosen as a control and resulted in a substitution at position 17. One of the altered toxins resulted from a site-directed mutation at position 54 and a mutation resulting from a replication error at position 51. The nomenclature of the mutant toxins indicates the position and type of substitution generated. For example, the codon for the serine residue at position 12 was changed to a codon for an alanine residue, and the resultant toxin is referred to as rSpeA1-S12A. Each of the mutant toxins and allelic forms was purified from *E. coli* as described in Materials and Methods and assayed by Western analysis for stability. With the exception of one mutant discussed below, all of the rSpeA forms migrated as one electrophoretic band (data not shown).

Mitogenic activities of rSpeA1 mutants. To assess the superantigenic activities of the recombinant toxins, each toxin was tested for its ability to stimulate human PBMCs. A representative experiment is shown in Fig. 3. The ability of rSpeA1-N20A to stimulate cells, as measured by [^{3}H]thymidine uptake, was slightly less than that of rSpeA1. rSpeA1-L24A showed a large decrease in mitogenic activity in comparison with rSpeA1.

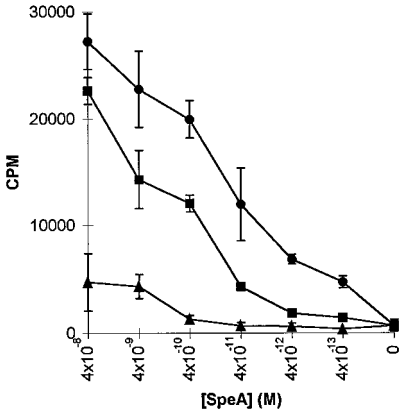

FIG. 3. Mitogenic activities of rSpeA mutants. rSpeA1-N20A (■) and rSpeA1-L24A (▲) were analyzed for the ability to stimulate human PBMCs relative to rSpeA1 (●) as described in Material and Methods. Values are the means of triplicate samples ± standard deviations. S.D.

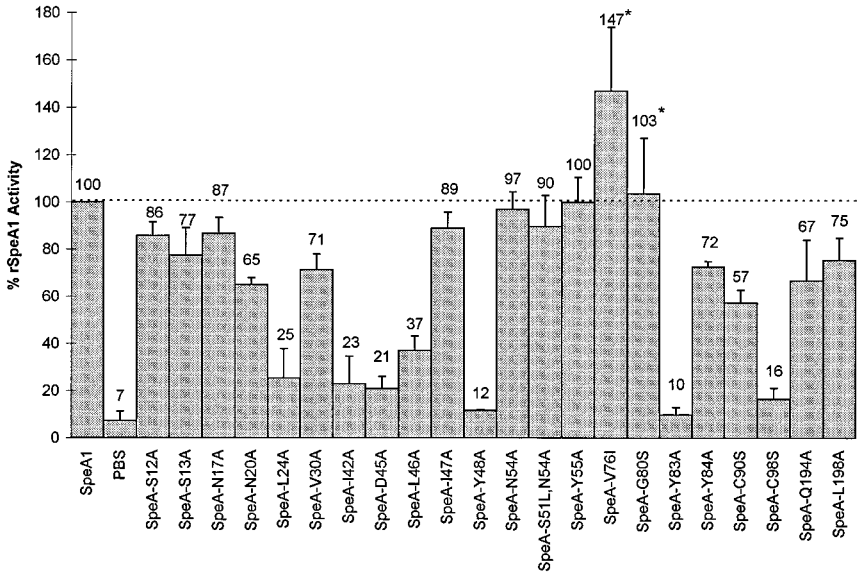

FIG. 4. Mitogenic activities of mutant and allelic forms of rSpeA compared with the activity of rSpeA1. The activity of rSpeA1 was set at 100%. PBMCs (10^5) obtained from healthy donors were incubated with 100 ng of recombinant toxins per ml for 3 days, pulsed with 1 μCi of [^{3}H]thymidine for 24 h, and harvested for liquid scintillation counting. Asterisks indicate the allelic forms rSpeA2 (rSpeA1-G80S) and rSpeA3 (rSpeA1-V76I). Mutant nomenclature is described in Materials and Methods. Values represent the means of three distinct experiments ± standard deviations. Values for allelic forms represent the means of five distinct experiments ± standard deviations.

The cell populations stimulated were analyzed by flow cytometry to determine the relative number of blast cells (data not shown). In all cases, the relative number of blast cells observed after stimulation with a specific toxin corresponded to the levels of that toxin's mitogenicity as measured by [^{3}H]thymidine uptake.

The relative activities of rSpeA1 and each of the rSpeA1 mutants are shown in Fig. 4. For this comparison, the activity of rSpeA1 was set at 100%, and the activities of the recombinant toxins are reported as percentages of the activity of rSpeA1. The mutant toxins can be placed into three categories based on a two-tailed paired *t*-test analysis of their mitogenic activities. The first category contains toxins with no statistical difference in activity relative to rSpeA1 ($P \geq 0.01$ compared with rSpeA1 and $P \leq 0.01$ compared with the negative control). Toxin forms with full activities include rSpeA1-S12A, -S13A, -N17A, -I47A, -N54A, -S51L-N54A, -Y55A, -Q194A, and -L198A and, with the exception of rSpeA1-Q194A, have at least 75% of the activity of rSpeA1. The *P* values define rSpeA1-Q194A as fully active even though the mean percent activity was 67. This was due to the large variation in mitogenic response of the various donor PBMCs to this mutant. Toxins in the second category are those that show intermediate levels of mitogenicity ($P \leq 0.01$ compared with rSpeA1 and $P \leq 0.01$ compared with the negative control). Toxin forms with intermediate activity, i.e., rSpeA1-N20A, -V30A, -L46A, -Y84A, and -C90S, have 25 to 75% of the activity of rSpeA1. Mutants in the third category show little or no activity ($P \leq 0.01$ compared with rSpeA1 and $P > 0.01$ compared with the negative control). These inactive forms, rSpeA1-L24A, -L42A, -D45A, -Y48A, -Y83A, -C98S, have less than or equal to 25% of the activity of rSpeA1. The implications of these mutations on the structure-function relationships of SpeA are addressed in Discussion.

Products of *speA* alleles differ in the ability to stimulate human PBMCs. Streptococcal strains carrying *speA2* or *speA3* have caused the majority of STSS episodes of the last decade (34). Toxin encoded by *speA3*, rSpeA3 (rSpeA-V76I), showed significantly greater mitogenic activity than rSpeA1 and stimulated PBMCs to a relative value of 147% ($P = 0.005$, $n = 5$ donors) (Fig. 4). rSpeA2 (rSpeA-G80S) stimulated immunocytes to a level 3% greater than that for rSpeA1, but this increase was not significant ($P = 0.75$, $n = 5$ donors).

A disulfide bond is required for superantigenic activity of rSpeA1. All allelic forms of SpeA contain three cysteine residues, and evidence for a disulfide bond in SpeA has been reported (33). SpeA3, isolated from *S. pyogenes*, analyzed under nonreducing and reducing conditions on SDS-PAGE shows a shift in electrophoretic mobility (data not shown), suggesting that this protein contains a disulfide bond. A similar shift in electrophoretic mobility also was observed with rSpeA1 (Fig. 5A, lanes 1 and 2). To determine which cysteine residues of rSpeA1 could be forming the disulfide bond, we generated mutant forms of the toxins that contained one of the three cysteine residues substituted with a serine residue. The resultant mutant toxins were examined by SDS-PAGE run under reducing and nonreducing conditions.

When the cysteine residue at position 90 was changed to serine (rSpeA1-C90S), the protein displayed the same migration shift as observed with rSpeA1 (Fig. 5A, lanes 3 and 4). This result indicated that the cysteine residue at position 90 was not involved in disulfide bond formation. In contrast, when the cysteine residue at position 98 was changed to serine (rSpeA1-C98S), the shift was no longer observed (Fig. 5A, lanes 5 and 6). Therefore, removal of the cysteine residue at position 98 prevented formation of the disulfide bond.

Substituting a serine residue for the cysteine residue at position 87 (rSpeA1-C87S) caused decreased stability of the toxin. SDS-PAGE analysis revealed several degradation products with molecular weights lower than the expected molecular weight of 25,787 (data not shown). One possible explanation for this instability is that the remaining cysteine residues at positions 90 and 98 formed an aberrant disulfide bond that changed the tertiary structure of the toxin, and this conformational change resulted in a protein that was more susceptible to

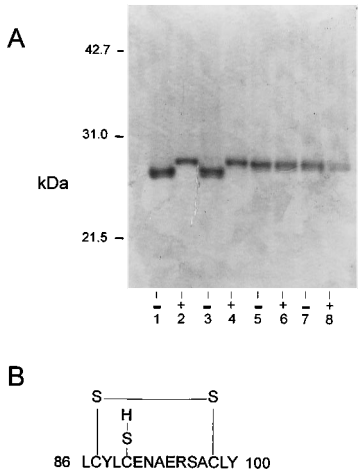

FIG. 5. Disulfide bond formation in SpeA. (A) Two-microgram aliquots of rSpeA1 (lanes 1 and 2), rSpeA1-C90S (lanes 3 and 4), rSpeA1-C98S (lanes 5 and 6), and rSpeA1-C87S,C90S (lanes 7 and 8) were mixed with 10 μl of gel loading buffer with (+) and without (−) 1% β-mercaptoethanol, boiled for 5 min, and analyzed by SDS–12% PAGE. Bands were visualized with Coomassie brilliant blue R250 (Bio-Rad). (B) Proposed residues involved in SpeA disulfide bond formation. The peptide shown begins at residue L-86 and ends with residue Y-100.

proteolytic cleavage. To test this hypothesis, the double cysteine mutant rSpeA1-C87S,C90S was created and expressed in *E. coli*. Mutating two of the three cysteine residues would prevent any aberrant disulfide bond formation that might make the toxin sensitive to proteases. When analyzed by SDS-PAGE, the purified toxin migrated as a single band of the correct molecular weight. As expected, there was no shift in the apparent molecular weight when the protein was run under nonreducing and reducing conditions (Fig. 5A, lanes 7 and 8). We conclude that cysteine residues 87 and 98 are linked in a disulfide bond (Fig. 5B).

To determine if the disulfide bond of SpeA was required for immunocyte stimulation, rSpeA1-C98S was analyzed for its ability to stimulate human PBMCs (Fig. 4). rSpeA1-C98S had little or no mitogenic activity. Mutation of cysteine residue 90 (rSpeA1-C90S), which was not involved in the disulfide bond, caused an approximate 50% decrease in toxin mitogenicity. Therefore, in SpeA the disulfide bond and residues near the disulfide bond play a critical role in the toxin's ability to function as a superantigen.

Binding of rSpeA1 to HLA-DQ molecules. An integral step in the stimulation of T cells by SpeA is the initial interaction of the toxin with the class II MHC molecule. SpeA binds to HLA-DQ molecules with a greater affinity than to either HLA-DR or HLA-DP (19). To determine the affinities of the allelic and mutant forms of SpeA for the DQ molecule, binding assays were performed between radiolabeled SpeA and L cells transfected with DQα- and DQβ-encoding cDNAs.

^{125}I-rSpeA1 bound to the L-cell DQ$^+$ transfectants (L54.1) (Fig. 6), and when increasing amounts of unlabeled competitor toxin were mixed with labeled toxin, the amount of ^{125}I-rSpeA1 bound to L54.1 cells decreased. There was a low level of binding observed between the radiolabeled toxin and untransfected control L cells (L66) (data not shown). However, this low-level binding could not be blocked with excess unlabeled toxin. When the binding of rSpeA1 to the DQ$^+$ L54.1 cells was examined by Scatchard analysis, both high-affinity binding and low-affinity binding were observed (Fig. 7). When binding of

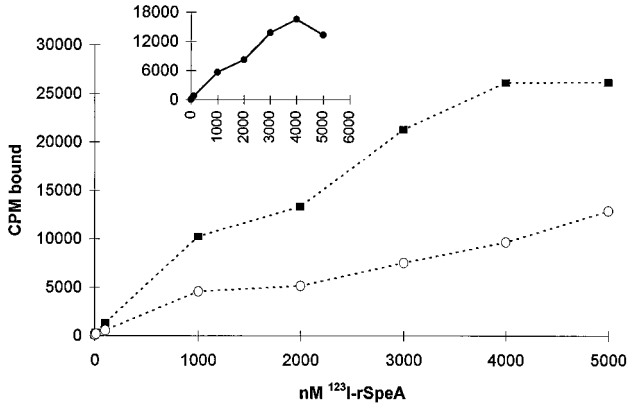

FIG. 6. Binding of rSpeA1 to DQ$^+$ L-cell transfectants. The DQ$^+$ L54.1 cells (10^6) were incubated for 4 h with increasing concentrations of radiolabeled rSpeA1 (■) or radiolabeled rSpeA1 and 100-fold-excess unlabeled toxin (○). Cell-associated toxin was measured as described in Material and Methods. The inset plots the specific binding of toxin to cells, as defined by the amount of labeled toxin bound at each concentration minus the amount of labeled toxin bound in the presence of excess unlabeled toxin (●). Specific binding datum points were used for the Scatchard analysis in Fig. 7.

rSpeA1 to the untransfected L66 cells was examined by Scatchard analysis, only low-affinity binding was observed (data not shown). Since high-affinity binding was detected only with the DQ-expressing cells, it was concluded that the high-affinity receptor on these cells was class II MHC. The Scatchard plot indicates that the K_d of this high-affinity interaction between rSpeA1 and HLA-DQ was 107 nM. The K_d of the lower-affinity interaction between rSpeA1 and the L cell surface was 1 μM.

Binding affinities of mutant and allelic forms of rSpeA for HLA-DQ molecules. The affinities of the mutant and allelic forms of SpeA1 for the HLA-DQ molecule were measured as a function of their abilities to inhibit binding of ^{125}I-rSpeA to the DQ$^+$ L54.1 cells. The inhibition constant (K_i) for rSpeA1 was determined to be 104 nM by Lineweaver-Burk analysis (Fig. 8), which is in close agreement with the K_d predicted by Scatchard analysis. Figure 8 also indicates that rSpeA1-L24A is a competitive inhibitor of rSpeA1 binding, with a K_i of 888 nM. The K_i for each of the various rSpeA mutants, as determined by Lineweaver-Burk analysis, is shown in Table 1. The four mutants with the least affinity for DQ molecules (rSpeA1-Y83A, -C98S, -D45A, and -L42A) have little or no mitogenic ability, confirming the importance of the class II MHC-toxin interaction for activity.

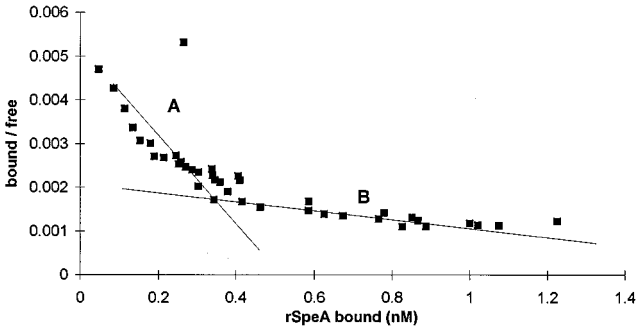

FIG. 7. Scatchard analysis of low- and high-affinity binding of rSpeA to DQ$^+$ L-cell transfectants. The DQ$^+$ L54.1 cells (10^6) were incubated with ^{125}I-rSpeA1 for 4 h at 37°C as described for Fig. 6. Line A represents high-affinity binding; line B represents low-affinity binding.

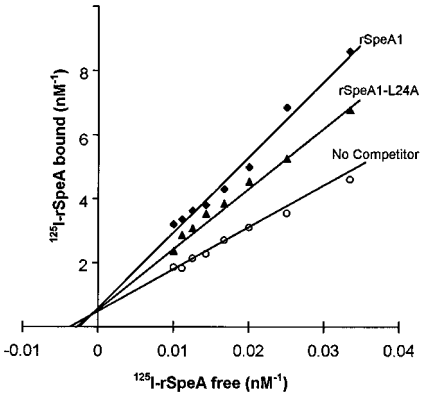

FIG. 8. Lineweaver-Burk analysis of rSpeA1 and mutant rSpeA1 binding to class II MHC transfectants. ^{125}I-rSpeA1 (30 to 100 nM) was incubated in 100 μl of DMEM–10% FCS–0.1% azide at 37°C for 4 h with either 150 nM unlabeled rSpeA1, 150 nM unlabeled rSpeA1-L24A, or no competitor. The values from assays with rSpeA1 and rSpeA1-L24A are displayed as examples of a high-affinity and an intermediate-affinity competitor, respectively. Cells were pelleted through an oil cushion, and bound toxin was quantitated by counting cells in a gamma counter. K_i values were calculated as described in Materials and Methods.

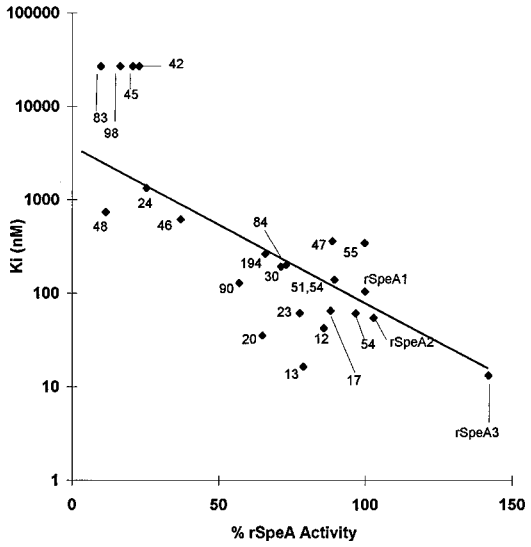

FIG. 9. Mitogenic activities of some rSpeA mutants correlate with class II binding affinities. Numbers identify amino acid residue altered in the mutant toxin.

rSpeA2 (K_i, 55 nM) and rSpeA3 (K_i, 13 nM) bound to DQ molecules with a higher affinity than rSpeA1 (Table 1). These toxins also stimulated PBMCs to a higher degree than rSpeA1 (Fig. 4). When the K_i of the interaction between the three allelic forms of SpeA and the DQ molecules was plotted as a function of mitogenic activity, a linear relationship was observed (Fig. 9). This result suggests that the mitogenic activity of each of the toxins increased as affinity for class II MHC increased.

The K_is of the mutant toxins were plotted as a function of their abilities to stimulate PBMCs (Fig. 9). For most of the toxins with measurable binding affinity, there was a direct correlation between the affinity for class II MHC and the ability to stimulate PBMCs. Certain toxins had DQ binding affinities equivalent to or greater than that of rSpeA1 yet showed intermediate levels of mitogenic activity (Fig. 9; rSpeA1-N20A, -V30A, and -C90S). This result suggests that these particular toxins can bind class II MHC but may be deficient in the ability to interact with the TCR. Future studies will assess the abilities of these toxins to stimulate T-cell subsets bearing specific Vβ chains.

TABLE 1. Affinities of rSpeA mutants and allelic forms for HLA-DQ

Toxin	K_i (nM)[a]
rSpeA1	104 ± 13
rSpeA-S12A	43 ± 15
rSpeA-S13A	16 ± 7
rSpeA-N17A	65 ± 24
rSpeA-N20A	36 ± 12
rSpeA-L24A	1,327 ± 552
rSpeA-V30A	192 ± 72
rSpeA-L42A	>27,000
rSpeA-D45A	>27,000
rSpeA-L46A	615 ± 123
rSpeA-I47A	358 ± 53
rSpeA-Y48A	733 ± 158
rSpeA-S51L,N54A	139 ± 76
rSpeA-N54A	61 ± 44
rSpeA-Y55A	342 ± 44
rSpeA3 (rSpeA-V761)	13 ± 1
rSpeA2 (rSpeA-G80S)	55 ± 38
rSpeA-Y83A	>27,000
rSpeA-Y84A	201 ± 31
rSpeA-C90S	129 ± 54
rSpeA-C98S	>27,000
rSpeA-Q194A	264 ± 121
rSpeA-L198A	62 ± 32

[a] Calculated as described in Materials and Methods. Values represent the means of three experiments ± standard deviations.

DISCUSSION

In recent years, many bacterial exotoxins have been shown to act as superantigens, and thus their toxicity results from their ability to overstimulate the host's immune system. Bacterial superantigens include the *S. aureus* toxins associated with food poisoning such as SEA, SEB, SEC1, SEC2, and SEC3; the staphylococcal toxins responsible for toxic shock syndrome, TSST-1 and TSST-2; and the streptococcal pyrogenic exotoxins SpeA, SpeB, and SpeC (9). SpeA is clearly an important member of this group because of its association with the development of STSS.

When the primary amino acid sequences of the various bacterial superantigens are compared, SpeA is found to be most closely related to SEB (6). The two toxins are approximately the same size and have 51% amino acid identity. Extensive mutational analysis of SEB (molecular weight, 28,478) has defined amino acid residues important for interaction with either class II MHC molecules or the TCR (22). It was this analysis that initially directed our research. The residues of SpeA mutated here were chosen on the basis of their identity to amino acids shown to be important for the superantigenic activity of SEB (with the exception of N-17, a nonconserved residue chosen as a control). In 1994, Jardetzky et al. reported the X-ray crystallographic structure of SEB bound to HLA-DR1 (20). This analysis defined 19 residues of SEB in direct contact with HLA-DR1.

A predicted ribbon structure of SpeA is shown in Fig. 10. Superimposed on this structure are the amino acid residues that were mutated in this report. The amino acid residues are

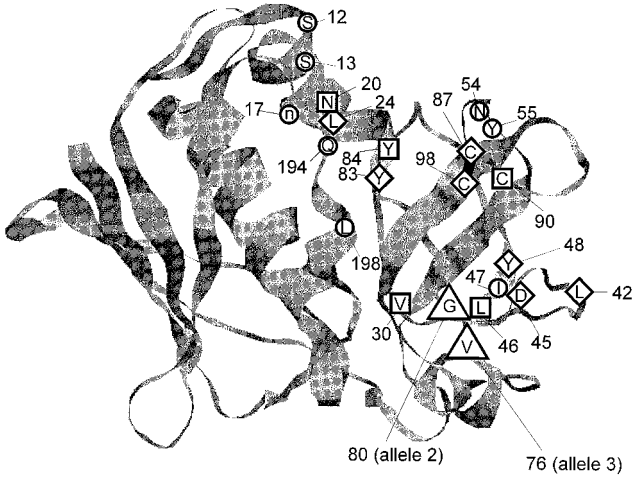

FIG. 10. Predicted ribbon structure of SpeA. The structure was generated and viewed as described in Materials and Methods. Amino acid residues highlighted are those substituted in this study. Numbers indicate positions of the residues in the primary structure of SpeA. Symbols: ○, position of substitution that resulted in full activity; □, the resultant toxin had an intermediate level of activity; ◇, substitution that resulted in a form with little or no activity; △, residue defining SpeA2 or SpeA3. Substitution of residue C-87 resulted in the production of an unstable toxin.

depicted in various shapes to highlight the effect of the substitution at this site on SpeA activity. A most striking finding is the pocket of residues, from L-42 to Y-48, that are clearly important for both mitogenic activity and a high-affinity interaction with the DQ molecule. The only substitution in this region that had little effect on activity, at position 47, was a conservative isoleucine-to-alanine change. The stretch of amino acids from positions 42 to 48 has a high degree of identity with a region in SEB (SEB residues 45 to 51) shown to be important for binding to class II MHC (20). The residues in SEB are in contact with or immediately adjacent to residues in contact with a predominantly hydrophobic depression on the DR1 α chain. Thus, we conclude that residues 42 to 48 of SpeA are probably in contact with or bordering residues in contact with, the DQ molecule. Similarly, Y-83 of SpeA aligns with Y-89 of SEB; Y-89 of SEB is thought to hydrogen bond with K-39 of the α chain of DR1. SpeA Y-83 may play a similar role in SpeA binding to the DQ molecule.

These data suggest that the class II binding domains of the two toxins are similar. Studies by Hartwig et al. show that binding of SEB to the HLA-DR molecules can be inhibited by SpeA (15), which supports this hypothesis. However, the two toxins show preferential binding to different HLA isotypes, and so there are presumably differences between the two in some of the contact residues. Jardetzky et al. (20) found that 17 residues of the DR1 α chain interact with SEB. Nine of these 17 residues, including K-39 mentioned above, are conserved between the α chains of DR1 and DQ (23). Thus, it is probable that the two toxins have similar but not identical binding pockets for class II MHC.

Our data indicated that L-24 of rSpeA was essential for high-affinity class II MHC binding. We cannot determine if this was due to a direct interaction or a structural or conformational requirement at this time. Hartwig and Fleischer reported five amino acid substitutions in SpeA that result in decreased binding to class II MHC: D-77, Y-100, D-146, R-150, and Y-160 (14). D-77 is adjacent to the residue at position 76 that differentiates SpeA1 from SpeA3, and Y-100 lies close to the critical residue C-98. It is not surprising that mutation of these residues would affect binding, since they are positioned close to residues shown here to be important for interaction with the HLA-DQ molecule. The other three mutations generated by Hartwig and Fleischer lie outside the class II MHC binding region defined here. Similar to the results for L-24, it cannot be concluded at this time if these substitutions disturb the native conformation of the toxin or if they are involved in a direct interaction with the HLA molecule. Hartwig and Fleischer (14) did not find an amino acid cluster of SpeA responsible for binding the class II MHC molecule, which probably reflects their choice of residues for substitution.

The significance of the mutants with approximate wild-type or greater than wild-type affinity for DQ yet a reduction in activity can only be speculated upon at this time. Presumably these mutants contain alterations in the amino acid residues important for interaction with the TCR. Our current experiments are to test this hypothesis. Although there is some overlap in the Vβ elements recognized by SEB and SpeA, each toxin binds to a distinct set of Vβ chains. Thus, we expect that the two toxins differ in their TCR binding sites. This divergence is reflected in the mutation at position 12 in SpeA that had little effect on the toxin's activity, whereas the analogous mutation in SEB decreased activity 10-fold (22).

SEB, SEA, and TSST-1 all have affinities for HLA molecules in the 10^{-7} M range, similar to that demonstrated here for SpeA (13, 30). The correlation of affinity of the mutant and allelic forms of SpeA with toxicity is clearly an approximation, as the superantigenic activity is a complex interaction that also involves binding to the TCR. However, it is an intriguing correlation and one that makes intuitive sense. Higher affinity for the class II molecule should lead to a higher percentage of productive toxin-HLA interactions. This in turn should lead to a higher probability of the complex activating the associated T cell. The correlation of affinity for the class II molecule with mitogenicity is also seen when the class II MHC binding affinities for SEB, SEA, and TSST-1 are plotted as a function of abilities of the toxins to activate T cells (30).

Scatchard analysis of rSpeA binding to the L-cell DQ^+ transfectants revealed both high- and low-affinity binding sites. The DQ^- L-cell line also showed low-affinity binding, suggesting that this low-affinity binding was to surface components that were not class II MHC molecules. Low-affinity interactions have been seen between other superantigens and components of eukaryotic cell surfaces. Beharka et al. found low-affinity binding of SEA and SEB to macrophages from transgenic mice with knockout mutations in the class II genes (5). The low-affinity binding of SEA was proposed to be either to one of several distinct membrane proteins or to class I MHC. Cantor et al. reported that SEC1 and SEE appear to interact with the integrin VLA-4 (11). However, it is not clear at this time if the low-affinity binding of SpeA to the L cells results from an interaction with either a class I MHC or integrin molecule or if it is due to a nonspecific interaction with the cell surface.

SpeA1-C98S, which did not form a disulfide bond, showed little or no mitogenicity, suggesting that SpeA requires a disulfide bond for activity. Eliminating the disulfide bond markedly diminished affinity for the DQ molecule, and possibly the correct formation of a disulfide loop is necessary for this interaction. The disulfide loop of SEB, which is in approximately the same position as that found in SpeA, covers the α1 α helix of the class II MHC DR1 molecule, masking residues that have been implicated in TCR recognition of peptide-MHC complexes (20). The similarity of the position of the SEB disulfide

loop to the position of the SpeA disulfide loop suggests that the SpeA loop may also be in close proximity to the class II MHC molecule and TCR. However, the disulfide loop in SEB is nine amino acid residues larger than that found in SpeA and thus might be able to have greater contact with the MHC molecule than the loop of SpeA. The amino acid substitutions of SpeA2 and SpeA3 lie adjacent to and on the amino-terminal side of the SpeA disulfide loop. Both of these substitutions increase the affinity of the protein for DQ, which suggests that this region of SpeA may be in close proximity to the MHC molecule.

A key finding of this report is that the toxin encoded by *speA3* was more mitogenic and showed a higher affinity for the HLA-DQ molecule than the toxin encoded by *speA1*. The amino acid substitution that generated SpeA3 was the only one constructed here that resulted in a more active toxin form. The single difference between rSpeA1 and rSpeA3 is a conservative valine-to-isoleucine substitution, and thus this large change in activity was unexpected. SpeA2 showed a slightly higher mitogenic activity and affinity for class II MHC than rSpeA1. Although the increase in both mitogenic activity and affinity for DQ is not large, we believe that it is significant for SpeA3. Since the interaction with class II MHC is one of the first in a complex chain reaction involving the expansion and stimulation of T cells and the production of cytokines, it can be envisioned that only a slight increase in activity could result in a large increase in toxicity and thus virulence to the host.

ACKNOWLEDGMENTS

We are grateful to Alan Bisno for introducing us to the group A streptococcus and to Robert Levy for many helpful discussions. We thank Sarah D'Orazio and Martin Flajnik for critical evaluation of the manuscript.

This work was supported by a grant from the Stanley Glaser Research Foundation and by an American Heart Association-Florida Affiliate Grant-in-Aid to C.M.C.

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Editor: V. A. Fischetti