Published in final edited form as:

Biochemistry. 2008 December 9; 47(49): 12995–13003. doi:10.1021/bi801468w.

Novel Toxic Shock Syndrome Toxin-1 Amino Acids Required for Biological Activity

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Abstract

Superantigens interact with T lymphocytes and macrophages to cause T lymphocyte proliferation and overwhelming cytokine production, which lead to toxic shock syndrome. *Staphylococcus aureus* superantigen toxic shock syndrome toxin-1 is a major cause of menstrual toxic shock syndrome. In general, superantigen-secreting *S. aureus* remain localized at the vaginal surface, and the superantigen must therefore penetrate the vaginal mucosa to interact with underlying immune cells to cause toxic shock syndrome. A dodecapeptide region (toxic shock syndrome toxin-1 amino acids F119-D130), relatively conserved among superantigens, has been implicated in superantigen penetration of epithelium. The purpose of this study was to determine amino acids within this dodecapeptide region that are required for interaction with vaginal epithelium. Alanine mutations were constructed in *S. aureus* toxic shock syndrome toxin-1 amino acids D120 to D130. All mutants maintained superantigenicity, and selected mutants were lethal when given intravenously to rabbits. Toxic shock syndrome toxin-1 induces interleukin-8 from immortalized human vaginal epithelial cells; however three toxin mutants (S127A, T128A, and D130A) induced low levels of interleukin-8 compared to wild type toxin. When carboxy-terminal mutants (S127A to D130A) were administered vaginally to rabbits, D130A was nonlethal, while S127A and T128A demonstrated delayed lethality compared to wild type toxin. In a porcine ex vivo permeability model, mutant D130A penetrated vaginal mucosa more quickly than wild type toxin. Toxic shock syndrome toxin-1 residue D130 may contribute to binding an epithelial receptor, which allows it to penetrate vaginal mucosa, induce interleukin-8, and cause toxic shock syndrome.

The staphylococcal superantigen toxic shock syndrome toxin-1 (TSST-1) is responsible for the majority of menstrual toxic shock syndrome (mTSS) cases and half of all nonmenstrual staphylococcal TSS cases (1,2). Staphylococcal enterotoxins (SEs) B and C are responsible for the other half of nonmenstrual TSS cases, whereas streptococcal pyrogenic exotoxin A (SPE A) and SPE C have been implicated as the main superantigens responsible for TSS cases caused by *Streptococcus pyogenes* (3-9). Superantigens were originally defined due to their unique mechanism of T lymphocyte stimulation (10). In the absence of normal antigen recognition, T cells are induced to proliferate when superantigens cross-bridge the variable region of the β chain of the T cell receptor ($V\beta$ -TCR) and major histocompatibility complex

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II (MHC II) on antigen presenting cells (11-15). This interaction results in the stimulation of 60-70% of T cells, which is referred to as T cell skewing (11). T cells and antigen presenting cells are also induced to produce an overwhelming amount of cytokines. These cytokines are responsible for the development of the symptoms of TSS, such as fever (interleukin [IL]-1 β), rash (IL-2 and interferon [IFN]- γ), and capillary leak (TNF- α and TNF- β), which lead to hypotension and eventual shock (16-21).

Although the superantigenicity of TSST-1 has been thoroughly studied, the toxin's effects on other cell types, such as epithelial cells, have not been well defined. In the case of mTSS this is especially important as toxin-producing *S. aureus* commonly remain localized on the tampon and mucosal surface, whereas the superantigen must penetrate the vaginal mucosa in order to induce the systemic effects seen in TSS. Small amounts of TSST-1 have been shown to penetrate porcine vaginal tissue, with the majority of the superantigen remaining localized within the tissue, perhaps serving as a reservoir (22). Porcine vaginal tissue is highly similar to human vaginal tissue in its cellular content and permeability; both tissues consist of nonkeratinized stratified squamous epithelium with intracellular lipids providing a permeability barrier (22-26). Our previous studies have demonstrated that TSST-1 causes an increase in production of proinflammatory cytokines and chemokines, such as TNF- α , IL-8, and MIP-3 α , from immortalized human vaginal epithelial cells (HVECs) (27). It was suggested that this initial inflammatory reaction may act to disrupt the mucosal barrier resulting in increased superantigen penetration.

Other studies have examined the ability of SEs to penetrate intestinal mucosa, in order to determine how these superantigens cause staphylococcal food poisoning. Shupp et al. identified a 10-amino-acid region of SEB (152-KKKVTAQELD-161) that could be targeted to inhibit transcytosis of the toxin across an intestinal epithelial monolayer (28). This region was originally identified by Wang et al. when the authors discovered inhibitory peptides generated against the superantigenic streptococcal pepsin-extracted type 5 M protein were similar in sequence to multiple streptococcal and staphylococcal superantigens (29). A 12-amino-acid (dodecapeptide) region can be found in most superantigens and is relatively conserved among superantigens in sequence similarity and structure (Figure 1). It is important to note that this region is also distinct from those regions required for V β -TCR and MHC II binding (30,31). Peptides generated against the dodecapeptide sequence in SEC are capable of neutralizing their ability to stimulate T cells (32). Arad et al. demonstrated that a dodecapeptide variant (YNKKKATVQELD) of this SEB region (original sequence TNKKKVTAQELD) could inhibit the SEB-induced expression of IL-2, IFN- γ , and TNF- α mRNA from peripheral blood mononuclear cells (PBMCs) (33-36). Rajagopalan et al., however, showed that peptides generated against the dodecapeptide sequence of SEB, as described by Arad et al., did not inhibit T cell proliferation in HLA class II transgenic mice (37). These authors also demonstrated that the peptides did not protect the transgenic mice from developing TSS.

We sought to identify the role of the dodecapeptide sequence in interactions with the vaginal mucosa due to the identification of this conserved peptide region in superantigen transcytosis of epithelial cell layers and production of cytokines from PBMCs. A study conducted previously in our laboratory indicated that the variant SEB dodecapeptide antagonist is capable of competitively inhibiting TSST-1-induced chemokine production from HVECs (27). Although the dodecapeptide sequence of TSST-1 is the most different among the superantigens, we chose to study it because of the exclusive association of TSST-1 with mTSS. The purpose of this study was to identify critical amino acids within the dodecapeptide of TSST-1 that are required to induce HVEC cytokine production and ultimately those that are involved in mTSS when the superantigen is administered vaginally.

Experimental Procedures

Generation of TSST-1 mutants

The plasmid pCE107 is a shuttle vector, consisting of pUC18 (origin of replication for *Escherichia coli*), pE194 (origin of replication for *S. aureus*) and *tstH*, which was mutated using the Stratagene Quick Change II site directed mutagenesis kit (La Jolla, CA). Primers were generated to change each amino acid in the TSST-1 dodecapeptide region (120-DKKQLAISTLD-130) to alanine (A125 was changed to serine; Table 1). The T128A mutant was generated previously (38). The primers were used in site-directed PCR per manufacturer protocol. Each PCR product was transformed into *E. coli* XL1-Blue supercompetent cells by heat shock and grown overnight in LB with 50 µg/ml ampicillin. Plasmids were collected from transformants using the Qiagen QIAprep Spin Miniprep kit (Valencia, CA). Plasmid preparations were electoporated into *S. aureus* RN4420 (200 ohms, 1.9 kV), and *S. aureus* were grown overnight in Bacto Todd Hewitt (Becton, Dickinson and Company, Sparks, MD) broth with 5 µg/ml erythromycin. Mutations in the dodecapeptide region were confirmed by DNA sequencing.

Toxin purification

Wild type TSST-1 and mutant toxins were isolated from *S. aureus* RN4220 (containing appropriate plasmids) after growth in beef heart media (2). Briefly, the cultures were treated with 80% ethanol (final concentration) at 4°C, the precipitates resolubilized in water, and toxins purified by two sets of isoelectric focusing. Isoelectric focusing was carried out by first using a pH gradient of 3.5 to 10, followed by another using a pH gradient of 6 to 8. The isoelectric point of TSST-1 is 7.2 (2). TSST-1 and mutant proteins were identified by double immunodiffusion based on reactivity with a specific polyclonal antibody generated against wild type toxin (39). Purity was confirmed by SDS-PAGE, which demonstrated single protein bands at a molecular weight of 22,000. Purified toxins were quantified using the BioRad protein assay (BioRad Co., Hercules, CA) with the superantigen SEB used for standard curve generation. Wild type and mutant toxins were also verified as homogeneous when samples were subjected to reversed-phase HPLC (Protein C4 column from Vydac [Hesperia, CA] 0 to 60% gradient of acetonitrile with 0.1% trifluoroacetic acid over a 30 min time period); proteins eluted as sharp single peaks.

Wild type TSST-1 and mutants A125S, S127A, and D130A were internally labeled with ³⁵S-methionine for porcine mucosa penetration studies. In previous labeling studies with TSST-1, approximately 10⁷ dpm/µg protein may be achieved. The protein purification is the same as that described above, except bacterial strains were cultured in 50 ml media containing 10 mCi ³⁵S-methionine.

Culture of HVECs

Immortalized HVECs, previously described by Peterson et al., were generated by transforming primary HVECs from a premenopausal woman with the E6/E7 genes of human papilloma virus 16 (27,40,41). Another vaginal epithelial cell line (CRL-2616) was obtained from American Type Culture Collection (ATCC, Manassas, VA) (42,43). Both cell lines were maintained in keratinocyte serum-free medium (KSFM; Gibco, Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract and epidermal growth factor as provided by the manufacturer and a 1% final volume of penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO) and amphotericin B (Fungizone; Gibco, Invitrogen). The cells were grown at 37°C in the presence of 7% CO₂. On days of experimentation, antimicrobials were not used since it has been observed that amphotericin B reduces cytokine production by HVECs.

Cytokine assay

Purified toxins (200, 100, 50, 20, 10, 1, and 0.1 µg/ml) were added to the cell culture medium and incubated with HVECs or CRL-2616 cells at 37°C in 7% CO₂ for 6 to 24 hours. At the conclusion of each experiment, media were collected and analyzed by ELISA using Quantikine® kits (R and D Systems, Minneapolis, MN). The production of IL-8 from the epithelial cells was determined, with the limit of detection being 3.5 pg/ml.

Superantigenicity assay

Wild type and mutant toxins were tested for superantigenicity in a four day assay (44). Cellular proliferation was measured based on DNA uptake of ³H-thymidine. Briefly, PBMCs were isolated from heparinized (100 units/ml) human blood by Ficoll-Hypaque sedimentation. Human blood was drawn in accordance with an approved University of Minnesota IRB protocol. PBMCs were cultured in RPMI 1640 medium (Lonza, Walkersville, MD) with 2% fetal calf serum (JRH Biosciences, Inc., Lenexa, KA), 200 µM L-glutamine (Sigma-Aldrich), and 1% penicillin-streptomycin (Sigma-Aldrich). Cells were incubated with toxin (1 µg-0.00001 µg per well) for three days. Eighteen hours prior to the completion of the experiment each well received 1 µCi of ³H-thymidine in 20 µl of medium. Cellular DNA was collected on glass-fiber filters using a MASH II® apparatus (Microbiological Associates, Bethesda, MD). A liquid scintillation counter (model LS; Beckman Instruments, Fullerton, CA) was used to measure thymidine uptake. Data were reported as the percent of wild type stimulation, based on average counts per minute (cpm) of three or four replicate samples.

Rabbit model experiments

Selected toxin mutants were tested in vivo using the rabbit model originally described by Kim and Watson (45) and Schlievert (46). This model tests the ability of superantigens to enhance shock caused by lipopolysaccharide (LPS) from *Salmonella enterica* serovar Typhimurium. Toxins, dissolved in phosphate-buffered saline (PBS; pH 7.2), were initially given to young adult American Dutch-belted rabbits (1.0-2.0 kg) as an intravenous (IV) dose (10 µg/kg) or an intravaginal dose (10 µg/animal). Toxins given IV were administered in the marginal ear veins. Intravaginal dosing was done as previously described (47). Briefly, rabbits were anesthetized with ketamine (25 mg/kg; Phoenix Pharmaceuticals, Inc., St. Joseph, MO) and xylazine (20 mg/kg; Phoenix Pharmaceuticals, Inc.) prior to insertion of catheters into rabbit vaginas. Toxins were delivered in 0.1 ml volumes. For all conditions, LPS (5 µg/kg; 1/100th lethal dose 50% endpoint of LPS alone) was administered IV in the marginal ear veins 4 hours after the initial superantigen doses, and the rabbits were monitored for 48 hours. Rabbits displaying signs of severe illness (failure to right themselves and exhibit escape behavior), or those remaining healthy at the conclusion of the experiment, were euthanized with Beuthanasia D (1 ml/kg, Schering-Plough Animal Health Corp., Union, NJ) according to the University of Minnesota IACUC requirement.

Penetration studies

An ex vivo porcine vaginal permeability model for superantigens has been previously described (22,24,27). Vaginal mucosal tissue was isolated from pigs at slaughter and used within 3 hours. Perfusion chambers were used to mount pieces of tissue (8-10 mm in diameter), with an exposed surface of 0.2 cm². PBS was continuously pumped through the lower chamber so that hourly cell-free samples of TSST-1 penetrating completely through the mucosa (up to 12 hours), could be taken. Seven replicates were used for each condition. Radioabeled ³⁵S-methionine TSST-1 (wild type or mutants, 15 µg/ml) was added to the upper chamber in PBS. Flux was calculated from the relationship $\text{flux} = Q/At$, where Q is the quantity of radiolabel traversing the tissue (disintegrations per minute) in time t (minutes) and A is the exposed epithelial surface area in square centimeters. Units of flux are therefore disintegrations per

minute per square centimeter per minute. Flux units were then converted to nanograms of toxin per square centimeter per minute based on the disintegrations per minute per nanogram of toxin.

Statistics

Significance of differences in two means were computed through determination of standard deviations and through Student's *t* test analysis of unpaired, normally distributed data. In studies of radiolabeled TSST-1 and selected mutants to penetrate intact ex vivo porcine vaginal tissue, the total amount of toxin (in ng) to penetrate through the tissue was calculated based on the quantity of radiolabel traversing the tissue (determined as disintegrations per minute) and corrected for using the disintegrations per minute of the original toxin solution. Values at each of the hourly sampling intervals were plotted against time and when there was no increase in value the chamber was assumed to have reached a steady state; 3-4 subsequent readings were then taken from which to calculate the mean for that chamber. For the seven replicate perfusion chambers a mean value and a measure of dispersion (standard error of the mean) was calculated. Different treatments were compared using a one-way analysis of variance and Duncan's multiple range test. Pairwise differences are considered significant if $p \leq 0.05$.

Results

Cytokine production in response to TSST-1 mutants

Two different immortalized HVEC lines were used to test the ability of TSST-1 mutants to stimulate production of IL-8. HVECs were incubated with toxins, supernates were collected, and IL-8 was measured by ELISA. Previous studies conducted in our laboratory showed that wild type TSST-1 elicits strong IL-8 responses from HVECs (27). Over a range of toxin doses (10 $\mu\text{g/ml}$ to 200 $\mu\text{g/ml}$), most mutants exhibited activity similar to wild type TSST-1 (Figure 2). Three of the mutants (Q123A, L124A and A125S) displayed significantly increased IL-8 production from HVECs (at one dose each) compared to wild type TSST-1 ($p < 0.05$). Two mutants (D120A and L124A) did not show expected dose response curves within the initial range of concentrations tested (10 $\mu\text{g/ml}$ to 200 $\mu\text{g/ml}$). These mutants were further diluted (1 $\mu\text{g/ml}$ and 0.1 $\mu\text{g/ml}$) in order to ascertain the fall-off of responsiveness to the toxins. TSST-1 mutations at the carboxyl end of the dodecapeptide sequence (S127A, T128A, and D130A) resulted in greatly reduced IL-8 stimulating activity. S127A at 200, 100, and 20 $\mu\text{g/ml}$ and T128A at 200 and 100 $\mu\text{g/ml}$ were statistically significantly different compared to wild type TSST-1 ($p < 0.05$). The last amino acid in the sequence, D130A, exhibited the most drastic decrease in IL-8 production as compared to wild type toxin; all concentrations were significantly lower than wild type TSST-1 ($p < 0.05$). D130A was verified to be non-toxic to the cells by trypan blue dye exclusion. A mutant towards the amino-terminus of the dodecapeptide, K121A, induced significantly lower levels of IL-8 from HVECs at low concentrations (20 and 10 $\mu\text{g/ml}$) compared to wild type TSST-1 ($p < 0.05$). A mutation in the equivalent residue in the dodecapeptide of streptococcal SPE A, K137A, also demonstrates a lower IL-8 response from HVECs (our unpublished data). The second cell line, CRL-2616, demonstrated a similar response to the mutants, however mutant S127A showed anomalous activity in this line (Figure 3). Even though a high dose of S127A (100 $\mu\text{g/ml}$) demonstrated wild type activity on these cells, a lower concentration of 10 $\mu\text{g/ml}$ was shown to have no activity compared to wild type toxin at this concentration. This confirmed that two separate lines of HVECs respond to TSST-1 by the production of IL-8 and that key residues within the dodecapeptide region may be responsible for inducing this action. A cartoon of the structure of TSST-1 is shown in Figure 4a. The dodecapeptide region is highlighted in yellow. Important residues in the dodecapeptide region are shown in Figure 4b (red residues are changes that led to decreased IL-8 production, whereas violet residues are changes that led to increased IL-8 production; not all residues are visible in this image).

Superantigenicity of TSST-1 mutants

Although the dodecapeptide sequence is distinct from both the V β -TCR and MHC II binding sites (30,48), the superantigenicity of the toxins was analyzed using a ^3H -thymidine assay, and each toxin was added to the wells at an initial concentration of 1 μg per well with 10-fold dilutions ranging down to 0.00001 μg per well. All mutants exhibited wild type activity, shown as a percentage of the wild type average cpm (Figure 5), with the exception of three surface exposed mutants, Q123A, L124A, and A125S, which had significantly higher activity at the 0.1 $\mu\text{g}/\text{ml}$ dose (all $p < 0.01$). Changes in these residues may act to enhance affinity for either the TCR or the MHC II receptor.

Effects of TSST-1 mutants in vivo

The four amino acids at the carboxy-terminal end of the dodecapeptide sequence were further examined since three of the four residues in this region were unable to induce wild type TSST-1 levels of IL-8 production from HVECs. In order to test the ability of the mutants to induce TSS in vivo, American Dutch-belted rabbits were used in two separate models of endotoxin enhancement (Table 2). Initially, each toxin was administered IV at 10 $\mu\text{g}/\text{kg}$ to two rabbits. Four hours later, LPS derived from *S. enterica* serovar Typhimurium was administered via the marginal ear vein at 5 $\mu\text{g}/\text{kg}$. Since this model delivers the toxin directly into the bloodstream, it tests solely the superantigenic activity of the mutants in vivo. Of the three mutants tested in this model (S127A, L129A, and D130A) all were determined to be lethal after 24 hours. The T128A mutant was tested originally by Murray et al. in a similar model and was also shown to be lethal (49). This again confirms that the carboxyl end of the dodecapeptide sequence is not required for superantigenicity. The second model of endotoxin enhancement involved giving young adult female rabbits the toxin intravaginally at a bolus dose of 10 μg . This was accomplished by inserting a catheter into the vaginal tract of each rabbit and administering the toxin in a 0.1ml volume. LPS was given four hours later as described above. Of the four mutants (S127A, T128A, L129A, and D130A), only L129A was fully lethal in this model as compared to wild type. This corresponded with the ability of L129A to elicit a wild type IL-8 response from HVECs. The D130A mutant, however, was incapable of causing any illness or lethality when given intravaginally to rabbits even though it maintained lethality when given IV. Mutants S127A and T128A were administered as a 10 μg dose and caused illness or lethality after 24 hours, however, the progression of disease was delayed compared to that caused by wild type TSST-1.

Penetration of porcine vaginal mucosa by TSST-1 mutants

The inability of D130A to cause TSS from the rabbit vaginal tract led us to believe that this TSST-1 mutant may be incapable of penetrating the mucosa in order to interact with underlying immune cells. To test this possibility, we used an ex vivo model of superantigen penetration that has been previously described (22,24,27). This model uses an automated perfusion chamber mounted with freshly harvested porcine vaginal mucosal tissue to assess the ability of superantigens to penetrate vaginal mucosa. Wild type TSST-1 and mutants D130A, S127A, and A125S were internally labeled with ^{35}S -methionine and added to the upper chambers (in replicates of 7) at a concentration of 15 $\mu\text{g}/\text{ml}$ in PBS. Hourly samples (up to 12 hours) were collected from the lower compartment and tested for radioactivity in order to determine the amount of superantigen penetrating the mucosa. Surprisingly, D130A and S127A penetrated the mucosa faster than both wild type toxin and A125S (Figure 6). There was a statistically significant difference in flux (ng/sq. cm/min) between both D130A and S127A and other toxins (p value < 0.05); there was no significant difference between wild type TSST-1 and A125S flux. The mean steady state fluxes for D130A and S127A were 4.11 ng/sq. cm/min and 3.35 ng/sq. cm/min, respectively, compared to 2.27 ng/sq. cm/min for A125S and 2.15 ng/sq. cm/min for wild type TSST-1. After 12 hours, only an average of 237 ng total had accumulated in

the lower chamber for wild type TSST-1. A125S was similar to wild type toxin in total accumulation of 239 ng, whereas S127A and D130A accumulated 394 ng and 488 ng total, respectively.

Discussion

The purpose of this study was to examine the role of a dodecapeptide region of staphylococcal TSST-1 (F119-D130), which is structurally conserved and variably conserved in primary amino acid sequence among superantigens (primary TSST-1 sequence is most different from other superantigens), in superantigenicity, vaginal mucosal penetration, and stimulation of IL-8 production from HVECs. Previous studies conducted in our laboratory demonstrated that TSST-1 induces proinflammatory cytokines and chemokines from HVECs (27). In addition, a SEB-like dodecapeptide antagonist (YNKKKATVQELD), in 10-fold excess amounts, competitively inhibited TSST-1-induced production of chemokines MIP-3 α and IL-8 from the same HVECs (27). These studies led us to hypothesize that residues within the dodecapeptide region are required for superantigen-induced cytokine production from the vaginal mucosal epithelium.

In order to address this possibility, we generated single-site alanine mutants along the TSST-1 dodecapeptide region, except changing Ala 125 to Ser, and analyzed the ability of the resultant mutants to induce IL-8 responses from HVECs. Alanine scanning, a technique often employed to change charged, potentially surface-exposed, amino acids to alanine, was used for mutant production; charged residues are hydrophilic, are able to form ion pairs and hydrogen bonds, and tend to be located on protein surfaces, and therefore may interact directly with host receptors. Additionally, alteration of non-exposed (buried) amino acid residues to alanine is likely to create a “hole” which is more easily tolerated structurally than the presence of a larger or charged buried amino acid. In this regard, all TSST-1 mutants retained approximately equal abilities to react with polyclonal antibodies raised against wild type TSST-1, indicating sufficient structural integrity was maintained for reactivity with highly specific immunoglobulins. Most mutants induced IL-8 responses from two HVEC lines similar to wild type TSST-1; however three mutants (S127A, T128A, and D130A) at the carboxy-terminal end of the dodecapeptide exhibited decreases in IL-8 production from the cells. We hypothesize that residues in this region are important for interactions with an undescribed epithelial cell receptor that leads to cytokine production; this receptor is distinct from the two known TSST-1 receptors, V β 2-TCR (31) and MHC II (30). HVECs do not express TCR, and fewer than 2% of HVECs, when cultured in KSFM, are positive for MHC II, indicating that TSST-1 is binding another receptor. Additionally, our unpublished studies indicate two TSST-1 mutants, Q136A which lacks ability to bind V β 2-TCR (31) and G31S/S32P which lacks ability to bind MHC II (31), stimulate wild type cytokine production by HVECs. The data indicate residues S127A, T128A, and D130A alter TSST-1 structure sufficiently such that interactions with this undescribed receptor are diminished. Residue T128 is surface exposed, seven amino acids away from residues important in TCR interaction with TSST-1 (H135 and Q136), at the base of a diagonal α -helix on the back of TSST-1 (in the standard view shown in Figure 3). This residue may directly contact the HVEC receptor. In contrast, S127 and D130 are non-exposed residues, and these may cause conformational changes that alter receptor interaction.

Three mutants in the central region of the dodecapeptide, Q123A, L124A and A125S, exhibited increased IL-8 production from HVECs. These same three mutants were more superantigenic at the 0.1 μ g/well dose, compared to wild type TSST-1. These amino acid residues appear to cause TSST-1 structural alterations that improve receptor interaction, possibly affecting both the epithelial cell receptor and one or both of V β 2-TCR or MHC II. All three of these residues are surface exposed and may directly contact the epithelial cell receptor. Studies are currently underway to identify the HVEC receptor.

Although the dodecapeptide region is distinct from the TSST-1 V β 2TCR and MHC II binding sites (30,31), we tested the superantigenicity of the mutants through assays of PBMC. All mutants exhibited wild type or greater (Q123A, L124A, and A125S) activity, which confirms that the dodecapeptide region is not required for superantigenicity, but may positively influence activity. It is potentially important that the HVEC response to TSST-1 requires greater amounts of superantigen (typically ≥ 10 μ g/ml) than superantigenicity ($\geq 10^{-3}$ μ g/ml) for responsiveness. We have demonstrated that TSST-1⁺ *S. aureus* strains may make toxin amounts in excess of 1000 μ g/ml in thin films, as *S. aureus* grows vaginally (unpublished observations). Thus, 10 μ g/ml is a biologically relevant concentration to stimulate epithelial cells. We hypothesize the reduced sensitivity of vaginal epithelial cells versus T cells and antigen presenting cells to TSST-1 is an evolutionary adaptation in humans. The reduced sensitivity of epithelial cells to TSST-1 suggests that foreign antigens, including TSST-1, do not stimulate massive cytokine responses in epithelial cells when only low antigen amounts or unrecognized antigens are encountered. Consistent with this, we recently identified four women with TSST-1 present vaginally in tampons during menstruation, lacking protective antibodies to TSST-1, and yet not developing mTSS (unpublished observation). In contrast, we expect that if T cells and antigen presenting cells were lining the mucosal surface, every woman would show massive cytokine production and develop mTSS upon exposure to even minute amounts of TSST-1.

We used two rabbit models of TSS development to assess the role of dodecapeptide mutants in vivo. The IV TSS model tests TSST-1 induction of proinflammatory cytokines, such as IL-1 β (induces fever), TNF- α and β (cause hypotension), and IL-2 and interferon- γ (result in rash), due to the interaction of V β 2-TCR⁺ T cells with macrophages, whereas the intravaginal model tests first the ability of TSST-1 to penetrate intact stratified mucosa, and subsequently superantigenicity. Four mutants at the carboxy-terminus of the dodecapeptide (S127A, T128A, L129A, and D130A) were tested in these two rabbit models of TSS. When given IV, all mutants displayed lethality comparable to wild type TSST-1 (T128A was tested previously and also found to be lethal (49)), consistent with their observed in vitro wild type superantigenicity. However, a differential lethal effect was seen when the mutants were administered intravaginally; L129A, which also maintained wild type cytokine-inducing activity on HVECs, maintained wild type lethality after intravaginal administration. Both S127A and T128A, two mutants that showed decreased ability to induce IL-8 from HVECs, caused lethality when given intravaginally; however the progression of disease was delayed compared to wild type TSST-1. D130A, which did not induce IL-8 from HVECs, was completely nonlethal when given intravaginally.

We hypothesized that the mutation at D130 altered the ability of TSST-1 to penetrate the vaginal mucosa. To examine penetration of mutants, we used an ex vivo porcine model that has been used previously to assess superantigen penetration of vaginal tissue (22,24,27). This model tests the ability of superantigens to penetrate vaginal stratified, squamous epithelial layers that lack tight junctions, in contrast to the single cell layer with tight junctions found in the intestinal tract. Shupp et al. previously showed that the dodecapeptide region is important for transcytosis across intestinal epithelial cells; however our studies demonstrate that this region is also important for interactions with stratified, squamous mucosa (28). Our collective studies suggest that superantigen transcytosis of the vaginal stratified, squamous epithelium is less important in superantigen penetration than superantigen induction of inflammation by causing cytokine production from epithelial cells to increase permeability, and resulting in superantigen movement around rather than through epithelial cells. We chose to study three mutants, D130A (no IL-8 from HVECs), S127A (low IL-8 from HVECs), A125S (high IL-8 from HVECs), along with wild type TSST-1. Unexpectedly, both D130A and S127A penetrated the porcine vaginal mucosa more quickly than either wild type TSST-1 or the mutant A125S ($p < 0.05$). Because this assay is based on counting radiolabeled TSST-1 as it moves through the tissue, it is possible that D130A and S127A were being degraded by mucosal

proteases, and therefore, we detected inactive toxin. Wild type TSST-1 is resistant to trypsin degradation; however some residue changes within the toxin are capable of rendering the protein sensitive to trypsin. In fact, Murray et al. demonstrated that one mutant within the dodecapeptide, T128A, is partially sensitive to trypsin degradation (49). This is interesting because this mutant also had reduced IL-8 activity when incubated with HVECs in our study. We analyzed the trypsin sensitivity of three mutants (D130A, S127A, A125S) as previously described (49), but did not see reduction in intact TSST-1 over 4 hours (data not shown), indicating trypsin degradation is not responsible for lack of activity at the epithelium.

It is unclear why the D130A mutant lacks toxicity when applied vaginally in the rabbit model when the same mutant penetrates porcine tissue faster than wild type TSST-1. If altering D130 results in a lower binding affinity of TSST-1 for epithelial cells, the mutant may simply move through the mucosa more quickly, consistent with our findings. However, the data suggest D130 must not be able access the circulation subsequent to penetration, since the mutant exhibits wild type lethality when administered IV. Further studies are needed to ascertain what role TSST-1 persistence in vaginal epithelium through interaction with the epithelial cell receptor plays in the development of mTSS.

Acknowledgements

This work was supported by USPHS research grant AI074283 from the National Institute of Allergy and Infectious Diseases.

Abbreviations used in text

TSST-1, (toxic shock syndrome toxin-1)
 TSS, (toxic shock syndrome)
 mTSS, (menstrual toxic shock syndrome)
 HVECs, (human vaginal epithelial cells)
 PBMCs, (peripheral blood mononuclear cells)
 SPE, (streptococcal pyrogenic exotoxin)
 SE, (staphylococcal enterotoxin)
 V β -TCR, (variable beta chain- T cell receptor)
 MHC II, (major histocompatibility complex II)
 IL, (interleukin)
 IFN, (interferon)
 TNF, (tumor necrosis factor)
 LPS, (lipopolysaccharide)
 KSFM, (keratinocyte serum-free medium)

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Superantigen	Dodecapeptide Sequence
SEB	T-N-K-K-K-V-T-A-Q-E-L-D
SEC	T- D -K-K- S -V-T-A-Q-E-L-D
SPE A	T-N-K-K- M -V-T-A-Q-E-L-D
TSST-1	F-D -K-K- Q-L-A-I-S-T -L-D

Figure 1.

Amino acid sequence of dodecapeptide region conserved among superantigens. Non-conserved peptides are shown in bold.

Figure 2.

IL-8 Dose-Dependent Response of HVECs to TSST-1 Mutants. Cells were incubated with toxin for 6 hours at concentrations of 200 µg/ml, 100 µg/ml, 20 µg/ml, and 10 µg/ml. Additional concentrations of 1 µg/ml and 0.1 µg/ml are shown for D120A, L124A, A125S, I126A, S127A. HVEC supernates were collected and assayed by ELISA for IL-8 production. (A) First six amino-terminal mutants. (B) Last five carboxy-terminal mutants. Results are reported as the difference from media only control. WT = wild type TSST-1.

Figure 3.

IL-8 Response of CRL-2616 cells to TSST-1 Mutants. Cells were incubated with wild type (WT) or mutant TSST-1 at 100 µg/ml for 24 hours. Cell supernates were then collected and analyzed for IL-8 production by ELISA. All results are reported as difference from a media only control. Carboxyl terminal mutants T128A and D130A show no reactivity from the cells, similar to that seen from the HVECs. A lower dose (10 µg/ml) is shown for WT and S127A toxins only to demonstrate that even though S127A at 100 µg/ml is highly reactive, its reactivity is quickly lost at 10 µg/ml.

Figure 4.

Structure of TSST-1. (A) Cartoon of TSST-1. The dodecapeptide region is shown in yellow. (B) Filled in structure of TSST-1. Amino acid residue changes that resulted in decreased IL-8 production from HVECs are shown in red, changes that maintained wild type IL-8 production are shown in yellow, and changes that caused an increase in IL-8 production are shown in violet (some residues are not visible in this image). Structures generated using PyMOL (DeLano Scientific LLC, South San Francisco, CA).

Figure 5. Superantigenicity of TSST-1 Mutants. Toxins were incubated with PBMCs for 4 days; ^3H -thymidine was added 18 hours prior to the conclusion of the experiment. Cellular DNA was harvested, and thymidine uptake was measured using a liquid scintillation counter. The average counts per minute were converted to percent of wild type. (A) Mutants D120A through A125S, (B) Mutants I126A through D130A.

Figure 6.

Penetration of TSST-1 Mutants Through Porcine Vaginal Mucosa. Wild type TSST-1 and mutants A125S, S127A, and D130A (15 μ g/ml each) were internally labeled with 35 S-methionine and added to the upper compartment of a perfusion chamber holding an 8-10mm piece of freshly harvested porcine vaginal tissue. PBS was continuously pumped through the lower compartment and collected hourly for 12 hours. Flow through was analyzed for radioactivity to determine the amount of penetrating toxin. Units of flux are in nanograms of toxin per square centimeter per minute and are an average of 7 replicates per condition.

Table 1
Primers used for site directed mutagenesis of *tstH*

D120A
Forward 5'-A AAG TAT TGG CCA AAG TTC GCT AAA AAA CAA TTA GC-3'
Reverse 5'- AT AGC TAA TTG TTT TTT AGC GAA CTT TGG CCA ATA C -3'
K121A
Forward 5'-TGG CCA AAG TTC GAT GCA AAA CAA TTA GCT ATA-3'
Reverse 5'-TAT AGC TAA TTG TTT TGC ATC GAA CTT TGG CCA-3'
K122A
Forward 5'- GG CCA AAG TTC GAT AAA GCA CAA TTA GCT ATA TCA AC-3'
Reverse 5'- AA AGT TGA TAT AGC TAA TTG TGC TTT ATC GAA CTT TG-3'
Q123A
Forward 5'- CA AAG TTC GAT AAA AAA GCA TTA GCT ATA TCA ACT TT-3'
Reverse 5'- TC TAA AGT TGA TAT AGC TAA TGC TTT TTT ATC GAA CT-3'
L124A
Forward 5'- AG TTC GAT AAA AAA CAA GCA GCT ATA TCA ACT TTA GA-3'
Reverse 5'-AA GTC TAA AGT TGA TAT AGC TGC TTG TTTTTT ATC GA-3'
A125S
Forward 5'- TC GAT AAA AAA CAA TTA AGT ATA TCA ACT TTA GAC TT-3'
Reverse 5'- TC AAA GTC TAA AGT TGA TAT ACT TAA TTG TTT TTT AT-3'
I126A
Forward 5'- AT AAA AAA CAA TTA GCT GCA TCA ACT TTA GAC TTT GA-3'
Reverse 5' AT TTC AAA GTC TAA AGT TGA TGC AGC TTA TTG TTT TT-3'
S127A
Forward 5'-AAA CAA TTA GCT ATA GCA ACT TTA GAC TTT GAA-3'
Reverse 5'-TTC AAA GTC TAA AGT TGC TAT AGC TAA TTG TTT-3'
L129A
Forward 5'-AA TTA GCT ATA TCA ACT GCA GAC TTT GAA ATT CGT CA-3'
Reverse 5'- GT ATG ACG AAT TTC AAA GTC TGC AGT TGA TAT AGC TA-3'
D130A
Forward 5'-GCT ATA TCA ACT TTA GCC TTT GAA ATT CGT CAT-3'
Reverse 5'-ATG ACG AAT TTC AAA GGC TAA AGT TGA TAT AGC-3'

Table 2

Rabbit models of TSS. Rabbits were given TSST-1 (wild type or mutant) either intravenously (IV) or intravaginally. For the IV condition, toxin (10 µg/kg) was administered via the marginal ear vein. For the intravaginal condition, toxin (10 µg bolus dose) was given via a catheter inserted in the vaginal tract. For both conditions, rabbits were given LPS (5 µg/kg) through the marginal ear vein four hours later. All toxins administered IV were lethal (T128A was tested by Murray et al. and was also found to be lethal (49); wild type TSST-1 was not used for this set of experiments since all mutants were found to be lethal). Only the D130A mutant was found to be nonlethal in the intravaginal model.

Mutant	Route	Dose	Survival at:		
			3 hours	24 hours	48 hours
D130A	IV	10 µg/kg	2/2	0/2	0/2
	Intravaginal	10 µg	3/3	3/3	3/3
L129A	IV	10 µg/kg	2/2	0/2	0/2
	Intravaginal	10 µg	1/3	0/3	0/3
T128A	Intravaginal	10 µg	3/3	1/3*	0/3
S127A	IV	10 µg/kg	2/2	0/2	0/2
	Intravaginal	10 µg	3/3	3/3*	1/3*
Wild type	Intravaginal	10 µg	1/3	0/3	0/3

* Animals remaining displayed symptoms of TSS and were euthanized prior to the end of the experiment.