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Cytolysins Augment Superantigen Penetration of Stratified Mucosa

Amanda J. Brosnahan,* Mary J. Mantz, † Christopher A. Squier, † Marnie L. Peterson, ‡ and Patrick M. Schlievert*†

Staphylococcus aureus and Streptococcus pyogenes colonize mucosal surfaces of the human body to cause disease. A group of virulence factors known as superantigens are produced by both of these organisms that allows them to cause serious diseases from the vaginal (staphylococci) or oral mucosa (streptococci) of the body. Superantigens interact with T cells and APCs to cause massive cytokine release to mediate the symptoms collectively known as toxic shock syndrome. In this study we demonstrate that another group of virulence factors, cytolsins, aid in the penetration of superantigens across vaginal mucosa as a representative nonkeratinized stratified squamous epithelial surface. The staphylococcal cytolsin α-toxin and the streptococcal cytolsin streptolysin O enhanced penetration of toxic shock syndrome toxin-1 and streptococcal pyrogenic exotoxin A, respectively, across porcine vaginal mucosa in an ex vivo model of superantigen penetration. Upon histological examination, both cytolsins caused damage to the uppermost layers of the vaginal tissue. In vitro evidence using immortalized human vaginal epithelial cells demonstrated that although both superantigens were proinflammatory, only the staphylococcal cytolsin α-toxin induced a strong immune response from the cells. Streptolysin O damaged and killed the cells quickly, allowing only a small release of IL-1β. Two separate models of superantigen penetration are proposed: staphylococcal α-toxin induces a strong proinflammatory response from epithelial cells to disrupt the mucosa enough to allow for enhanced penetration of toxic shock syndrome toxin-1, whereas streptolysin O directly damages the mucosa to allow for penetration of streptococcal pyrogenic exotoxin A and possibly viable streptococci. The Journal of Immunology, 2009, 182: 2364–2373.


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Abbreviations used in this paper: TSS, toxic shock syndrome; HVEC, human vaginal epithelial cell; IEF, isoelectric focusing; mTSS, menstrual TSS; SEB, staphylococcal enterotoxin B; SLO, streptolysin O; SPE, streptococcal pyrogenic exotoxin; TSST-1, TSS toxin-1.

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Porcine vagina ex vivo has also been used to analyze the ability of TSST-1 to penetrate the mucosa. Ex vivo porcine tissue is an excellent model of human vaginal tissue; vaginal tissue from both human and pig is a nonkeratinized stratified squamous epithelium with intercellular lipids, including ceramides, glucosyl ceramides, and cholesterol located in the surface layers (34, 37–43). As tight junctions are not present between the cells of the vaginal mucosa, the intercellular lipids constitute a permeability barrier. TSST-1 labeled with [35S]methionine has been shown to cross the ex vivo porcine vaginal mucosa with significant amounts of toxin remaining within the tissue (44). Studies by Peterson et al. demonstrated that the penetration of TSST-1 is enhanced by the presence of heat-killed and live St. aureus (45). Live bacteria were better able to enhance penetration of TSST-1 than heat-killed bacteria, indicating that secreted factors made by the bacteria may be contributing to the disruption of the mucosal barrier. The authors suggested that cytolsins may play a role in this process due to their probable ability to provoke inflammatory responses.

The main objective of the present study was to determine the role of two cytolsins, streptolysin O (SLO) and α-toxin, in the penetration of superantigens across vaginal mucosa as a model of nonkeratinized stratified squamous epithelium. SLO is a thiol-activated toxin made by Str. pyogenes that belongs to a group of cytolsins known to bind cholesterol and form pores in the membranes of eukaryotic cells (46). Seventy to 80 monomers of SLO oligomerize on cell membranes to form large pores up to 30 nm in diameter that can be lethal for most cell types (47, 48). α-Toxin, made by St. aureus, is a heptamer pore-forming toxin that creates small (2.6 nm diameter) pores in eukaryotic membranes (49). Many species-specific cell types can be killed by α-toxin, including erythrocytes, platelets, mononuclear immune cells, endothelial cells, and epithelial cells. In cellular damage, both cytolsins can elicit cytokine responses from epithelial cells that may contribute to disruption of the mucosa. Dragneva et al. showed that low amounts of α-toxin induce secretion of IL-8 from epithelial cells and monocytes (50). Nonlethal doses of SLO stimulate the release of IL-6 and IL-8 from HCaT human keratinocytes, whereas a SLO-deficient Str. pyogenes mutant induces lower levels of IL-1α, IL-6, and IL-8 from these cells compared with wild-type bacteria (51, 52).

Superantigens may also interact with cells of the mucosa to induce inflammation that may independently disrupt the mucosal barrier. A few studies have shown that superantigens can induce cytokine responses from other cell types. The staphylococcal superantigen TSST-1 has been shown to bind both endothelial and epithelial cells and in some cases is internalized by the cells (53–56). More recently, it was demonstrated that TSST-1 and SEB induce cytokine responses from epithelial cells. TSST-1 incubated with vaginal epithelial cells induces TNF-α, MIP-3α, and IL-8 and induces TNF-α and IL-8 production from bronchial epithelial cells (45, 57). SEB has been shown to induce an IL-8 response from nasal epithelial cells and to alter the permeability of rabbit maxillary sinus epithelium, indicating that the enterotoxin can specifically interact with epithelial cells (58, 59). Rajagopalan et al. and Herz et al. have shown that SEB also induces systemic inflammatory responses when administered from mucosal surfaces, including nasal, conjunctival, and vaginal mucosae (60–64). Rajagopalan et al. also demonstrated a similar effect when the streptococcal superantigen SPE A was administered nasally to HLA-transgenic mice; however, not much is known about the ability of streptococcal superantigens to stimulate a cytokine response from the epithelium itself (64). Peterson et al. demonstrated that, like TSST-1, SPE A can induce proinflammatory cytokine production from human vaginal epithelial cells (HVECs) (45). The second objective of the present study was to evaluate the ability of superantigens (TSST-1 and SPE A) and cytolsins (α-toxin and SLO) to induce cytokine responses from HVECs.

In the present study we show that α-toxin and SLO act to enhance penetration of TSST-1 and SPE A, respectively, across ex vivo porcine vaginal mucosa in a model of superantigen penetration. Both cytolsins cause localized damage and inflammation in the ex vivo porcine tissue; however, purified cytolsins added to HVECs induced cell death and damage to different extents. Superantigens incubated with HVECs induce proinflammatory cytokine and chemokine responses from the cells, but these responses are altered when cytolsin is present. Based on these data, we propose two separate models for streptococcal and staphylococcal superantigen penetration across vaginal mucosa.

**Materials and Methods**

**Penetration studies**

An ex vivo porcine vaginal permeability model for superantigens has been previously described (34, 44, 45). Briefly, porcine vaginal mucosa was isolated from pigs at slaughter and used within 3 h of harvest. Tissue discs (8–10 mm in diameter) were mounted between two halves of continuous perfusion chambers, exposing ~0.2 cm² of the epithelial surface to the donor compartment. Internally labeled 35S-TSST-1 (10–50 μg/ml) in the absence or presence of α-toxin (5 or 50 μg/ml) or 35S-SPE A (40 μg/ml) in the absence or presence of SLO (5 μg/ml or 50 μg/ml) was added to the upper compartment in PBS. DTT (Roche Diagnostics) at a final concentration of 10 mM was added to all specimens receiving SLO to maintain a reducing environment for the oxygen-labile cytolsin. Seven replicates were used for each condition. PBS was continuously pumped through the lower compartment as a collection fluid for up to eight hourly samples and the dpm of the samples were counted in a scintillation counter. An aliquot of each radio labeled solution was also counted to determine the dpm per nanogram of toxin applied. The total amount of toxin to traverse the tissue was determined by converting the dpm of the samples to nanograms of toxin using the dpm/nanogram conversion factor determined for the radiolabeled toxin solutions (44). Tissue discs from each treatment group were removed from the chambers at the conclusion of the experiment, fixed in formalin, wax embedded, cross-sectioned, and stained with H&E for histological examination. One specimen from each group was snap frozen in liquid nitrogen and cut in cross sections at 14 μm, and the sections were placed on x-ray film and developed to visualize radioactive toxin remaining in the tissue.

Dutch-belted rabbits are highly sensitive to TSST-1 when administered continuously for 7 days in s.c. miniosmotic pumps (16). However, rabbit susceptibility to TSST-1 is most easily measured by the capacity of TSST-1 to synergize with LPS up to 10⁵-fold through acceleration of cytokine release; the animals succumb within 48 h (65) whether TSST-1 is administered i.v. or intravaginally (36). Thus, we performed an experiment in female rabbits to assess the ability of α-toxin to facilitate TSST-1 vaginal penetration following intravaginal administration of TSST-1 plus α-toxin, followed 4 h later by i.v. LPS. We have experimentally determined that i.v. administration of 0.01 μg/kg TSST-1 to rabbits followed at 4 h with 50 μg/kg LPS is 100% lethal, whereas administration of these agents is not lethal when TSST-1 is administered intravaginally (0.01 μg/kg) followed i.v. with LPS (50 μg/kg). Incidentally, the lethal dose of TSST-1 alone, given either per rectum or intravenously, is ~3.5 mg/kg and for LPS i.v. is ~>500 μg/kg. α-Toxin (0.05 μg/kg) is not lethal to rabbits whether given i.v. or intravaginally. Thus, we compared the ability of 0.01 μg/kg TSST-1 with or without α-toxin (both intravaginal) to synergize with LPS (50 μg/kg) i.v. 4 h later. Intravaginal administrations of TSST-1 and α-toxin in PBS were made through catheters threaded into the vaginas of rabbits after anesthesia with ketamine and xylazine (36); LPS (Salmonella enterica serovar Typhimurium) was given i.v. through the marginal veins of the animals were monitored for development of TSS over 48 h. In agreement with rules of the University of Minnesota Institutional Animal Care and Use Committee, rabbits that failed to exhibit escape behavior and could not right themselves were considered to have lethal TSS and were euthanized.

In an additional experiment, Str. pyogenes strain MNBU (M-type 3 clinical isolate; 8×10⁷ CFU or S. aureus strain MN (vaginal TSS isolate; 1×10⁶ CFU) were added to the upper chamber in the absence or presence of SLO and α-toxin (5 μg/ml each), respectively. DTT was added to all conditions receiving SLO at a final concentration of 10 mM. Four to five
replicates were used for each condition. Perfusion was collected every 2 h for 8 h. Samples were concentrated to obtain a bacterial pellet (14,000 rpm for 5 min) and then resuspended in 400 μL of PBS and plated out on Todd-Hewitt agar to determine the amount of bacteria that penetrated the epithelium. The total number of CFU was determined for each sample and averaged among the replicates for each time point. The total number of CFU at the conclusion of the experiment were determined by adding the average total CFU number for each time point (2, 4, 6, and 8 h).

Toxin preparation

The superantigens TSST-1 and SPE A were purified as previously described (22, 66). Briefly, TSST-1 was isolated from Staphylococcus aureus strain RN4220 (pCE107) and SPE A was isolated from Bacillus subtilis strain IS75 (pJS103 MiniKCN) grown in beef heart medium (67). The cultures were precipitated with ethanol at 4°C, the precipitate was resolubilized in water, and toxin was purified by isoelectric focusing (IEF). IEF was conducted in two phases; the first phase used a pH gradient of 3.5–10, followed by another phase using a pH gradient based on the isoelectric point of the toxin. The isoelectric point of TSST-1 is 7.2 so the second IEF phase used a gradient of 6–8, whereas the isoelectric point of SPE A is 5.2 and the second IEF phase used a gradient of 4–6 (22, 68). Each superantigen was identified in a double immunodiffusion assay based on its specific reactivity with polyclonal Ab generated against the exotoxin (69). Purified TSST-1 and SPE A were quantified by SDS-PAGE, which demonstrated a single protein band at a m.w. of 22,000 (TSST-1) or 26,000 (SPE A). Purified toxins were quantified by radioimmunoassay, confirming that the bacteria may be making an exoprotein that augments superantigen penetration. It has been previously suggested that the bacteria may be making an exoprotein that augments superantigen penetration. TSST-1 was added at 10 μg/ml whereas SPE A was added at a higher concentration of 40 μg/ml based on previous studies done on rabbit vaginal mucosa, which indicated that SPE A was not able to penetrate the mucosa as well as TSST-1 (36). An additional set of chambers was used to determine the penetration of only 10 μg/ml SPE A; this set demonstrated that SPE A penetrates the porcine vaginal mucosa just as well as TSST-1. SEM is for 3–7 replicates. Statistical difference was calculated using Student’s unpaired t test with normally distributed data (compared to amount of superantigen able to penetrate on its own).

Table I. Cytolysins augment penetration of superantigens

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration of Toxin (total ng ± SEM) after 8 h</th>
<th>Statistical Difference when Cytolysin Is Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSST-1 (10 μg/ml)</td>
<td>25 ± 2.33</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>TSST-1 (10 μg/ml) plus α-toxin (5 μg/ml)</td>
<td>39 ± 6.78</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>TSST-1 (10 μg/ml) plus α-toxin (50 μg/ml)</td>
<td>55 ± 11.59</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>SPE A (40 μg/ml)</td>
<td>112 ± 21.05</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>SPE A (40 μg/ml) plus SLO (5 μg/ml)</td>
<td>176 ± 12.67</td>
<td>NS</td>
</tr>
<tr>
<td>SPE A (40 μg/ml) plus SLO (50 μg/ml)</td>
<td>150 ± 7.33</td>
<td></td>
</tr>
<tr>
<td>SPE A (10 μg/ml)</td>
<td>28 ± 6.23</td>
<td></td>
</tr>
</tbody>
</table>

*S Superantigens (TSST-1 or SPE A) were internally labeled with [35S]methionine and applied to the epithelial surface of fresh porcine vaginal mucosa mounted in perfusion chambers in the absence or presence of cytolsin α-toxin or SLO (5 or 50 μg/ml). In both cases, cytolsins acted to disrupt the mucosa and augment superantigen penetration. TSST-1 was added at 10 μg/ml whereas SPE A was added at a higher concentration of 40 μg/ml based on previous studies done on rabbit vaginal mucosa, which indicated that SPE A was not able to penetrate the mucosa as well as TSST-1 (36). An additional set of chambers was used to determine the penetration of only 10 μg/ml SPE A; this set demonstrated that SPE A penetrates the porcine vaginal mucosa just as well as TSST-1. SEM is for 3–7 replicates. Statistical difference was calculated using Student’s unpaired t test with normally distributed data (compared to amount of superantigen able to penetrate on its own).

Uric acid assay

Uric acid release from injured cells was measured using the QuantiChrom kit available from BioAssay Systems. This assay uses the compound 2,4,6-tripyrild-1-triazine, which forms a blue-colored complex with iron only in the presence of uric acid. The intensity of the color change is proportional to the amount of uric acid present in the sample.

Trypan blue cell staining

After incubation with exotoxins, HVECs were rinsed and subjected to trypan blue (Invitrogen) treatment and centrifugation (200 × g for 5 min) to obtain a cell pellet. Cells were then resuspended in 0.5 ml of keratinocyte serum-free medium and 0.1 ml 0.4% trypan blue staining solution (Sigma-Aldrich) for 15 min. HVECs were counted using a hemacytometer and survival percentages were calculated.

Statistics

In all cases where statistical analysis was necessary, mean values and SEs of the mean were determined. Statistical difference between means was determined using the Student’s unpaired t test with normally distributed data. Fishers exact test was used to assess differences in TSS survival rates between experimental and control rabbit groups.

Results

Cytolysins augment penetration of superantigens across vaginal mucosa

An ex vivo porcine model of superantigen penetration of vaginal mucosa has been used previously to demonstrate that staphylococcal TSST-1 penetrates the mucosa in small amounts, with most remaining on the tissue (45). In the presence of Staphylococcus aureus, however, the amount of superantigen that penetrates is greatly increased. When comparing the amount of TSST-1 that penetrates in the presence of live or heat-killed Staphylococcus aureus, more superantigen can penetrate vaginal mucosa when live bacteria are present, suggesting that the bacteria may be making an exoprotein that augments superantigen penetration. It has been previously suggested by our laboratory that cytolsins, such as α-toxin, may act to disrupt the vaginal mucosa to allow for better penetration of TSST-1. It is also possible that this may be the case for streptococcal superantigens; therefore we chose to examine the ability of the streptococcal cytolsin SLO to augment penetration of SPE A.

Table I shows the penetration through porcine vaginal tissue of radiolabeled 35S-TSST-1 and 35S-SPE A in the absence or presence of cytolsins α-toxin and SLO, respectively. In both cases,
The presence of cytolysins increased the total amount of superantigen that penetrated the vaginal mucosa. In the case of TSST-1 and α-toxin, both concentrations of cytolysin significantly enhanced the amount of TSST-1 that penetrated the epithelium (p < 0.05). In the case of SPE A and SLO, however, only the lower dose of SLO (5 μg/ml) was able to significantly augment the penetration of SPE A (p < 0.05). A larger starting concentration of SPE A was used (40 μg/ml compared with 10 μg/ml TSST-1), because previous studies using rabbit vaginal mucosa showed that SPE A was unable to penetrate as well as TSST-1 (36); however, we also measured the amount of SPE A at 10 μg/ml that penetrated the porcine tissue and found that SPE A was able to penetrate as well as TSST-1 in this model.

At the conclusion of each experiment, tissue specimens were sectioned and stained with H&E to visualize damage to the mucosa. α-Toxin at both the low (5 μg/ml) and high (50 μg/ml) doses damaged the surface layers of the vaginal mucosa, with obvious sloughing of the epithelium seen at the higher dose (Fig. 1). SLO also damaged the mucosa at both concentrations (5 and 50 μg/ml) with significant intraepithelial separation again seen at the higher dose (Fig. 2). SPE A alone, in contrast, did not damage the epithelium, compared with a PBS only control.

To determine where in the tissue the nonpenetrating superantigen was located, tissue sections were placed on x-ray film and developed. This allowed us to visualize radioactivity remaining on or within the tissue, and it indicated that the majority of the radiolabeled superantigen was “trapped” in the uppermost epithelial layers of mucosa (Fig. 3). It is interesting to note that even though cytolysins augment superantigen penetration, the majority of superantigen still remained in the surface layers of the tissue and did not penetrate throughout. It is important to remember that only microgram amounts of superantigen are required to cause TSS in humans (73); therefore, although the differences between the amount of superantigen that penetrates in the absence or presence of cytolysin may appear small, the differences may explain the development of TSS or the simple clearance of superantigen without TSS.

Superantigens and cytolysins induce different cytokine responses from HVECs

Although cytolysins were shown to damage the vaginal mucosa, it was possible that both superantigens and cytolysins induced proinflammatory responses that disrupted the barrier and facilitated greater penetration of molecules across the vaginal surface. In fact, our laboratory has previously shown that TSST-1 (100 μg/ml) can induce the production of proinflammatory cytokines and chemokines (IL-8, MIP-3α, and TNF-α) from an immortalized HVEC line (45). However, in this study we show that a lower dose of TSST-1 (10 vs 100 μg/ml) induced only a small amount of IL-1β and IL-6 from HVECs (Fig. 4). When α-toxin was incubated with HVECs at two concentrations (50 and 5 μg/ml) it induced all cytokines and chemokines tested (IL-1β, TNF-α, IL-6, and IL-8) in a dose-dependent manner, with the exception of MIP-3α, which was not detected. When TSST-1 (10 μg/ml) was coincubated with α-toxin (5 or 50 μg/ml) on the HVECs, very little IL-1β or TNF-α was detected, but IL-6 and IL-8 were induced to a greater extent, indicating synergy between TSST-1 and α-toxin. In general, when both TSST-1 and α-toxin were incubated with the cells, the extent of the immune response was dependent on the concentration of α-toxin.

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FIGURE 1. α-Toxin disrupts vaginal mucosa. TSST-1 (10 μg/ml) and α-toxin at 5 μg/ml (A) or 50 μg/ml (B) were applied to ex vivo porcine vagina and incubated for 8 h. Tissue was sectioned and stained with H&E for histological examination. Both concentrations of α-toxin appear to disrupt the surface layers of the vaginal epithelium, with the higher concentration causing sloughing of the uppermost epithelial layers.

FIGURE 2. SLO damages vaginal tissue. SPE A (40 μg/ml) in the absence or presence of SLO (5 or 50 μg/ml) was applied to ex vivo porcine vagina for 8 h. Tissues were sectioned and stained with H&E for histological examination. A, SPE A alone does not damage the mucosa. B and C, A lower dose of SLO (5 μg/ml) shows damage to the epithelial surface (B), but more distinct damage, including intraepithelial separation, can be seen at the higher dose (50 μg/ml) (C). D, Control tissue that has been incubated in PBS alone for 8 h.

FIGURE 3. Most SPE A remains in the tissue. SPE A was radiolabeled with [35S]methionine before application to the porcine vagina ex vivo. Whole pieces of tissue were sectioned and exposed to x-ray film to determine where the superantigen remained in the tissue. In all conditions SPE A primarily remained in the uppermost layers of the epithelium, which can be seen on the x-ray film (top). Stained tissue sections are also shown for reference (bottom).
α-toxin present. An experiment was also conducted using a higher dose of TSST-1 (100 μg/ml) with both concentrations of α-toxin (5 or 50 μg/ml) that showed the same trend; however the overall response was reduced compared with that seen with a lower dose of TSST-1 (data not shown).

In contrast to the proinflammatory response to α-toxin from the HVECs, SLO alone (10 μg/ml) induced only a low level of IL-1β from the cells (Fig. 5). Lower doses of SLO (1 and 0.1 μg/ml) were shown to elicit similar but dose-dependent responses from the HVECs (data not shown). SPE A, like TSST-1 (at 100 μg/ml), induced strong IL-8 and MIP-3α responses from the cells, and this response was found to increase over time (data not shown). SPE A also induced IL-6, which was only detected in response to the lower TSST-1 concentration. A lower concentration of SPE A (10 μg/ml) demonstrated a similar trend to that seen with the higher dose. When SPE A (100 or 10 μg/ml) and SLO (10 μg/ml or 1 μg/ml) were incubated with the HVECs, only an IL-1β response was seen. This response was stronger than that to SLO alone; however, it is important to note that any cytokines and chemokines induced by SPE A alone were no longer detected.

Cytolsins cause different amounts of damage to the HVECs

The cytolsins may have been directly damaging or killing the epithelial cells, which may have triggered the inflammatory response seen. Three methods were used to assess cellular damage: trypan blue staining of dead cells, measurement of uric acid release from damaged cells, and comparison of total cell numbers in the absence or presence of cytolsin after 6 h. The release of uric acid from cells and tissues has been shown to correlate with cellular damage, and extracellular uric acid acts as a danger signal to the immune system by triggering dendritic cells that phagocytose the molecules to become activated (74). The high dose of α-toxin (50

FIGURE 4. α-Toxin induces a proinflammatory cytokine and chemokine response from HVECs. Cells were incubated with TSST-1 (A), α-toxin (B), or combinations of both for 6 h (C); cell culture supernatants were collected and assayed by ELISA for IL-1β, TNF-α, IL-6, IL-8, and MIP-3α. Peterson et al. (45) demonstrated that a higher concentration of TSST-1 (100 μg/ml) can induce IL-8 and MIP-3α from HVECs, as well as low levels of TNF-α. In the present study we show that a lower concentration (10 μg/ml) of TSST-1 induced only low levels of IL-1β and IL-6 (A), whereas α-toxin at two doses (50 and 5 μg/ml) induced a wider range of proinflammatory cytokines and chemokines (B). When administered simultaneously to the HVECs at three different combinations of concentrations (high SLO at 10 μg/ml and high SPE A at 100 μg/ml; high SLO at 10 μg/ml and low SPE A at 10 μg/ml; and low SLO at 1 μg/ml and high SPE A at 100 μg/ml), only IL-1β was detected and all cytokines and chemokines induced by SPE A were no longer detectable (C). All concentrations are given as a difference from a medium only or DTT only control. Error bars represent the SEM.

FIGURE 5. SPE A elicits a proinflammatory response from HVECs, but only IL-1β can be measured when SLO is present. SPE A and/or SLO were added to HVECs for 6 h; cell culture supernatants were collected and analyzed by ELISA for cytokine and chemokine production by the cells. SLO alone (10 μg/ml) induced only a small IL-1β response from the cells (A), whereas SPE A alone (100 μg/ml) induced large amounts of IL-6, IL-8, and MIP-3α (B). When administered simultaneously to the HVECs at three different combinations of concentrations (high SLO at 10 μg/ml and high SPE A at 100 μg/ml; high SLO at 10 μg/ml and low SPE A at 10 μg/ml; and low SLO at 1 μg/ml and high SPE A at 100 μg/ml), only IL-1β was detected and all cytokines and chemokines induced by SPE A were no longer detectable (C). All concentrations are given as a difference from a medium only or DTT only control. Error bars represent the SEM.
μg/ml) killed the HVECs only after 6 h, as seen by only a 10% survival rate when the total cell number was compared with that of a medium only control (Fig. 6, yellow hatched column), but no uric acid release was detected under any condition. Of those cells remaining adherent after 6 h in the presence of the high dose of α-toxin, only 33% survived (Fig. 6, yellow column). The lower dose of α-toxin (5 μg/ml) did not induce uric acid release from the cells at any time point. SLO, in contrast, induced uric acid release from the HVECs after 2 h, but lower levels of uric acid were detected at 4 and 6 h, indicating that uric acid is not stable in the cellular medium. The percentage of cells remaining after 6 h is shown by a blue hatched column as determined by a difference in total cell number compared with a medium only control. α-Toxin did not induce uric acid release from the cells at any time point, but the high dose (50 μg/ml) killed the majority of the cells after 6 h (yellow columns). The percentage of total cells remaining after 6 h is shown by a yellow hatched column. Cell death due to α-toxin was not seen at 2 (orange columns) or 4 (violet columns) h. The low dose of α-toxin (5 μg/ml) did not induce uric acid release or kill the cells. A medium only control is shown (white column) to demonstrate that cells typically do not release uric acid. Error bars on uric acid data represent the SEM.

FIGURE 6. SLO induces uric acid release from dead and damaged cells, but only a high dose of α-toxin kills the cells without inducing the release of uric acid. To assess the ability of the cytolysins to damage or kill HVECs, uric acid release (●) from the cells was measured as an indicator of cell damage (74) and trypan blue staining (columns) was done to determine the percentage of cells alive after each time point. SLO (10 and 1 μg/ml) and the DTT only control induced a strong uric acid release from the cells after only 2 h (red columns), indicating cellular damage. At 4 h (green columns), not as much uric acid was detected but only 53% of the cells were still alive as indicated by trypan blue staining. After 6 h, the cells still adherent to the flask were alive (blue columns) and very little uric acid could be detected, indicating that the uric acid is not stable in the cellular medium. The percentage of cells remaining after 6 h is shown by a blue hatched column as determined by a difference in total cell number compared with a medium only control. α-Toxin did not induce uric acid release from the cells at any time point, but the high dose (50 μg/ml) killed the majority of the cells after 6 h (yellow columns). The percentage of total cells remaining after 6 h is shown by a yellow hatched column. Cell death due to α-toxin was not seen at 2 (orange columns) or 4 (violet columns) h. The low dose of α-toxin (5 μg/ml) did not induce uric acid release or kill the cells. A medium only control is shown (white column) to demonstrate that cells typically do not release uric acid. Error bars on uric acid data represent the SEM.

FIGURE 7. SLO enhances the ability of Str. pyogenes to penetrate vaginal epithelium, whereas α-toxin does not. Str. pyogenes (8 × 10⁹ CFU) and Staphylococcus aureus (1 × 10⁹ CFU) were added to ex vivo porcine vagina and incubated for 8 h in the absence or presence of SLO and α-toxin (5 μg/ml each), respectively. Perfusate was collected every 2 h, concentrated, and plated on Todd-Hewitt agar plates to determine the amount of viable bacteria that penetrated the epithelium. Total bacterial concentration was determined by adding total CFU for each 2-h sample up to 8 h. Although Str. pyogenes was able to penetrate the epithelium on its own, the presence of SLO allowed for a significantly higher amount of bacteria to penetrate (p < 0.05). There was no significant difference between the amounts of Staphylococcus aureus that were able to penetrate in the absence or presence of α-toxin.
Table II. α-Toxin enhances the lethality of TSST-1 in rabbits

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rabbit Lethality after 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSST-1 (0.01 μg/kg)</td>
<td>0/4</td>
</tr>
<tr>
<td>TSST-1 (0.01 μg/kg) plus α-toxin (0.05 μg/kg)</td>
<td>4/4 (p &lt; 0.03)</td>
</tr>
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* A rabbit model of TSS that monitors the ability of TSST-1 to synergize with lipopolysaccharide to cause shock through the acceleration of cytokine release was used to assess the ability of α-toxin to enhance the penetration of TSST-1 across rabbit vaginal epithelium. TSST-1 (0.01 μg/kg) in the absence or presence of α-toxin (0.05 μg/kg) was administered intravaginally to young adult female Dutch-belted rabbits; 4 h later LPS (50 μg/kg) was administered i.v. and rabbits were monitored for 48 h for signs of TSS. Fisher’s exact test was used to assess differences in TSS survival rates between experimental and control rabbit groups.

absence or presence of SLO and α-toxin, respectively. Fig. 7 shows the amount of bacteria that penetrated the epithelium after 8 h. SLO was clearly able to enhance the ability of *Sta. pyogenes* to penetrate the vaginal epithelium; the amount of bacteria that penetrated was significantly higher than all other conditions (p < 0.05). *Sta. pyogenes* alone was better able to penetrate than *Sta. aureus* alone but was only significantly higher in concentration after 2 h (p < 0.001). No bacteria were detected to penetrate when α-toxin was present with *Sta. aureus*; however, there was no significant difference between the amounts of *Sta. aureus* that penetrated in the absence or presence of the cytolysin.

α-toxin augments penetration of TSST-1 in a rabbit vaginal model of TSS

Unlike the relatively thick human and porcine vaginal mucosa, the rabbit vaginal mucosal is only 3–5 epithelial cell layers in thickness. The ability of α-toxin to facilitate TSST-1 penetration was assessed following intravaginal TSST-1 with or without intravaginal α-toxin (Table II). Development of lethal TSS was assessed by the synergy of cytokine production due to subsequent i.v. LPS given 4 h after intravaginal TSST-1 with or without intravaginal α-toxin. Rabbits that received intravaginal TSST-1 plus intravaginal α-toxin succumbed to TSS (4/4) when challenged with LPS. In contrast, rabbits that received intravaginal TSST-1 alone did not succumb to TSS (0/4) when challenged with LPS (p < 0.03), suggesting the cytolysin facilitated TSST-1 penetration of the vaginal mucosa.

Discussion

Both *Sta. aureus* and *Str. pyogenes* can initiate TSS from nonkeratinized stratified squamous mucosal surfaces; *Sta. aureus* causes mTSS while remaining localized on vaginal epithelia, whereas *Str. pyogenes* induces TSS often associated with initial oral mucosal colonization and subsequent penetration of the organism and superantigens systemically. In the case of mTSS, *Sta. aureus* locally produces the superantigen TSST-1 which must then penetrate the mucosal barrier to interact with adaptive immune cells to induce the cascade of events that leads to shock. Previous research done in our laboratory demonstrated that live bacteria could enhance superantigen penetration better than heat-killed bacteria, indicating that live *Sta. aureus* may be actively secreting factors that aid in disrupting the barrier (45). The main purpose of this study was to determine the role of cytolysins in the penetration of superantigens across vaginal mucosa. Although *Str. pyogenes* can occasionally be found vaginally and induce TSS from this site (75), it is more commonly found in the oral mucosa. This study used only vaginal mucosa as a representative nonkeratinized stratified squamous epithelium to examine both staphylococcal and streptococcal superantigen penetration; however, previous studies have indicated a high similarity between vaginal and oral epithelia in both structure and function, including permeability (41, 43).

In this study, we demonstrated that both the staphylococcal cytolysin α-toxin and the streptococcal cytolysin SLO are capable of augmenting the penetration of their respective superantigens. In the case of α-toxin, there was a dose-dependent increase in the amount of TSST-1 that was able to penetrate ex vivo porcine vaginal tissue. A greater concentration of SLO, in contrast, did not seem to further enhance the ability of SPE A to penetrate. This may be due to the difference in the amount of monomers required to form a pore for each cytolysin. SLO requires between 70 and 80 monomers, whereas α-toxin only requires seven; therefore, SLO may cause site-specific damage to the mucosa whereas α-toxin can cause a broader range of damage with an increase in concentration. Histological examination showed disruption of the mucosa due to the presence of cytolysin in both cases. Although more superantigen was able to penetrate through the mucosa when cytolysin was present, we demonstrated for SPE A that the majority of the radiolabeled toxin remains in the uppermost layers of the tissue. This entrapment of superantigen in what appears to be the mucosal epithelium may serve as a reservoir for additional toxin during the infection process. We have previously demonstrated a reservoir effect in porcine oral mucosa with TGF-β3, a molecule similar in size to the superantigens (76).

Although damage to the mucosa was seen in histological staining of tissues exposed to cytolysins, it was unclear whether that was due to direct damage by the cytolysins or was the result of an inflammatory process caused by the cytolysins. To clarify this, we examined the ability of α-toxin and SLO to induce proinflammatory responses from immortalized epithelial cells. Although α-toxin was shown to induce a strong proinflammatory response from the cells, SLO only induced a low level of IL-1β. To address the possibility that cytolysins and superantigens act to synergistically induce an inflammatory response from epithelial cells, we measured the amount of cytokines produced in response to both α-toxin and TSST-1 or SLO and SPE A. Although α-toxin alone was proinflammatory, there was a differential immune response to α-toxin and TSST-1 when administered simultaneously. In the presence of α-toxin and TSST-1, the cytokines IL-1β and TNF-α were no longer detected but higher amounts of IL-6 and IL-8 were seen, indicating some synergy between the two toxins. In contrast, there was a stronger IL-1β response to both SLO and SPE A; however the cytokines and chemokines induced by SPE A alone were not detected when SLO was present.

To examine the direct damage to HVECs caused by α-toxin and SLO, we measured uric acid release (an indicator of cell damage and a known “danger” signal for the immune system; Ref. 74) and stained the cells with trypan blue to identify dead cells; we also compared total cell numbers in the presence and absence of cytolysin after 6 h. Interestingly, α-toxin did not cause uric acid release from the cells at 2, 4 or 6 h, but the high concentration of α-toxin (50 μg/ml) killed ~90% of the cells after 6 h. The strong proinflammatory response we saw from the cells incubated with α-toxin indicated that the cells are able to make and secrete large amounts of cytokines and chemokines before the majority of them succumb to the cytotoxic effects of the toxin. SLO killed almost half of the cells by 4 h, but cell damage was evident after only 2 h as indicated by a strong release of uric acid from the cells. The cells remaining adherent after 6 h incubation with SLO were still alive; however, most of the cells were no longer adherent (only 38% of the cells remained). We believe that the low IL-1β response that SLO induces from HVECs is due to the release of preformed IL-1β from the cells and not due to de novo synthesis of new cytokines, as we
saw an IL-1β response as early as 2 h after SLO was added to the cells (data not shown).

The ability of SLO to damage epithelial cells without eliciting a strong proinflammatory response led us to hypothesize that SLO may also act to augment penetration of \textit{Str. pyogenes} across the epithelium. Using ex vivo porcine vaginal epithelium, we demonstrated that SLO does in fact enhance the ability of \textit{Str. pyogenes} to penetrate the epithelium. In the case of \textit{Sta. aureus}, however, the presence of α-toxin does not increase the ability of the bacteria to penetrate the vaginal tissue. This is interesting because \textit{Sta. aureus} typically remains on the vaginal surface in cases of menstrual TSS, whereas \textit{Str. pyogenes} is often found in the bloodstream in cases of streptococcal TSS. Therefore, it is possible that SLO causes enough damage to the mucosa to allow for penetration of both SPE A and \textit{Str. pyogenes}, whereas α-toxin only acts to enhance penetration of TSST-1 across the epithelium. This is consistent with in vivo experiments conducted in rabbits that demonstrated the ability of α-toxin to enhance penetration of TSST-1 across the vaginal epithelium to cause lethal TSS.

Based on these results we propose two separate models of penetration for staphylococcal and streptococcal superantigens (Fig. 8). In the case of \textit{Sta. aureus} infections, both α-toxin and TSST-1 are secreted at the vaginal surface. α-Toxin induces a strong proinflammatory response from HVECs without necessarily damaging the epithelium (unless at high concentrations). The localized inflammation caused by α-toxin acts to disrupt the barrier, allowing TSST-1 to penetrate the epithelium and interact with active epithelial cells in the underlying layers to induce a proinflammatory response of its own. Chemokines elicited by TSST-1 recruit adaptive immune cells to the area, which the superantigen can then interact with to induce T cell proliferation and massive cytokine production, leading to TSS. In the case of \textit{Str. pyogenes} infections, it follows that both SPE A and SLO are made on mucosal surfaces. SLO directly damages the epithelium without inducing a strong cytokine response from the cells. The damage caused by SLO allows SPE A to better penetrate the epithelium to gain access to lower level epithelial cells, which are more active and therefore can be stimulated to produce proinflammatory cytokines by the superantigen. At this point, the model becomes similar to that proposed for \textit{Sta. aureus}, in which adaptive immune cells are recruited to the underlying layers of the mucosa and the superantigen starts the cytokine cascade that eventually leads to TSS. The damage caused by SLO in the absence of a strong inflammatory response may also allow \textit{Str. pyogenes} to penetrate the mucosal barrier, which may help to explain why \textit{Str. pyogenes} often becomes systemic in cases of streptococcal TSS, whereas \textit{Sta. aureus} remains localized on the vaginal surface during cases of mTSS.

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**FIGURE 8.** Two different proposed models for penetration of staphylococcal and streptococcal superantigen penetration of vaginal mucosa. A, In the case of \textit{Sta. aureus} infections, both α-toxin and TSST-1 are secreted at the vaginal surface. α-Toxin induces a strong proinflammatory response from HVECs without necessarily damaging the epithelium (unless at high concentrations). The localized inflammation caused by α-toxin acts to disrupt the barrier, allowing TSST-1 to penetrate the epithelium and interact with active epithelial cells in the underlying layers to induce a proinflammatory response of its own. Chemokines elicited by TSST-1 recruit adaptive immune cells to the area, which the superantigen can then interact with to induce T cell proliferation and massive cytokine production, leading to TSS. B, In the case of \textit{Str. pyogenes} infections, it follows that both SPE A and SLO are made on mucosal surfaces. SLO directly damages the epithelium without inducing a strong cytokine response from the cells. The damage caused by SLO allows SPE A to better penetrate the epithelium to gain access to lower level epithelial cells, which are more active and therefore can be stimulated to produce proinflammatory cytokines by the superantigen. At this point, the model becomes similar to that proposed for \textit{Sta. aureus}, in which adaptive immune cells are recruited to the underlying layers of the mucosa and the superantigen starts the cytokine cascade that eventually leads to TSS. The damage caused by SLO in the absence of a strong inflammatory response may also allow \textit{Str. pyogenes} to penetrate the mucosal barrier, which may help to explain why \textit{Str. pyogenes} often becomes systemic in cases of streptococcal TSS, whereas \textit{Sta. aureus} remains localized on the vaginal surface during cases of mTSS.
mucosa. This is important, because in streptococcal TSS Str. pyogenes is often found in the bloodstream compared with nTSS in which Staphylococcus aureus remains localized on the vaginal surface. The lack of inflammatory response to SLO may provide a way for the Str. pyogenes to rapidly traverse the superficial mucosal barrier before an appropriate immune response can be mounted against the bacteria.

We showed that the majority of radiolabeled SPE A remains in the uppermost layers of the mucosa, which may act as a reservoir for additional superantigen during infection. Other work done in our laboratory has implicated that superantigens may be “sequenced” in the mucosa through interactions with a receptor on epithelial cells (45, 80). Significant interaction between the superantigen and epithelial cells may be required to induce an immune response sufficient to initiate the cascade of events that leads to TSS. Therefore, it is possible that cytolsins are only secreted at the mucosal surface to create an initial disruption of the barrier that subsequently allows superantigens to gain access to the more active epithelial cells in the lower layers of the mucosa. There, the interaction between superantigens and epithelium leads to production of chemokines required to recruit an ample number of adaptive immune cells to the site of infection. At this point, the superantigens are able to cross-bridge the TCR of T cells and the MHC II of APCs so as to induce massive cytokine production, which eventually will lead to TSS.

In sum, these studies have demonstrated the ability of staphylococcal and streptococcal cytolsins to augment penetration of superantigens through porcine vaginal tissue in an ex vivo model of superantigen penetration. The cytolsins were shown to cause damage to the mucosa, particularly the outermost layers of tissue. In vitro human cell studies demonstrated that while both superantigens (TSST-1 and SPE A) were capable of inducing cytokine and chemokine production from HVECs, only the cytolysin α-toxin was proinflammatory when incubated with the cells. SLO acted quickly to damage the cells, and we believe that only preformed IL-1β was released. A high concentration of α-toxin killed the cells; however this process was slow enough to allow for production and secretion of various cytokines and chemokines before cell death. Finally, we proposed two distinct models of superantigen penetration for Str. aureus and Str. pyogenes that involved disruption of the mucosal tissue through an inflammatory reaction to α-toxin and through direct cellular damage by SLO, both of which lead to enhanced superantigen penetration.

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Disclosures
The authors have no financial conflict of interest.

References


