Transcytosis of Staphylococcal Superantigen Toxins

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Summary

Staphylococcus aureus produces a set of exotoxins including staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin 1 (TSST-1) which cause food poisoning and toxic shock syndrome in human beings and other species (1-4). These toxins are intermediate molecular weight proteins (22-30 kD) that also act as superantigens (SAgs) and toxins. Although their mode of action as SAgs is well understood, little is known about how they enter the body via the intestine and cause food poisoning. To examine this problem we used an in vitro culture system to study the capacity of class II MHC-negative human intestinal epithelial cells (Caco-2) to transcytose several staphylococcal toxins. We found that Caco-2 cells are capable of dose-dependent, facilitated transcytosis of SEB and TSST-1, but not SEA. We extended these studies in vivo in mice by showing that ingested SEB appears in the blood more efficiently than SEA. Our data suggest that these toxins can cross the epithelium in an immunologically intact form. These results may have important implications for the pathogenesis of food poisoning.

Materials and Methods

Cell Lines. The human adenocarcinoma cell line, Caco-2 (18), was obtained from American Type Culture Collection (Rockville, MD). Two T cell hybridomas were used in these studies.

Abbreviations used in this paper: HRP, horseradish peroxidase; SAg, superantigen; SE, staphylococcal enterotoxins; TEER, transepithelial electrical resistance; TSST-1, toxic shock syndrome 1.
2B10.D20 (VP8.2+ ) was derived from a B10.D2 mouse and recognizes SEB presented by a variety of class II MHC molecules (Kappler, J., and P. Marrack, unpublished data). SKC (VP3+) was derived from a B10.BR mouse and responds to SEA, again presented by class II MHC molecules (19). The HLA-DR1 transfected fibroblast, DAP-DR1, and the B lymphoblastoid cell line, LG2, were used as APCs for SEB and SEA (20–22).

Toxins. SEA and TSST-1 were obtained from Toxin Technology (Madison, WI). Recombinant wild-type and mutant SEB proteins were affinity purified from lysates of E. coli or S. cerevisiae containing the appropriate toxins as previously described (22).

Mice. B10. BR animals were bred in the animal care facility at the National Jewish Center (Denver, CO).

mAbs. In this study, three anti-SEB mAbs (B327, 2B33, and B344; reference 23), two anti-SEA mAbs (A116.3 and A108.6; Kappler, J., and P. Marrack, unpublished data), and two anti-TSSST-1 mAbs (T327H1.1 and 477L; reference 24) were used. The anti-TSSST-1 mAbs were a gift of M. Matsuura (NEXstar Pharmaceuticals, Lakewood, CO). Detecting mAbs were biotinylated using NHS-S-S-biotin (Sigma Chemical Co., St. Louis, MO). Biotin conjugated anti-TCR Vb mAbs, KJ25 (anti-Vb3), and F23.1 (Vb8) were purchased from Pharmingen (San Diego, CA). The anti-CD4 mAb GK1.5 (25) was FITC labeled using a standard protocol.

Preparation and Selection of Complete Monolayers. Caco-2 cells were grown to confluence on plastic flasks (225 cm²) and subcultured every week using trypsin-EDTA (GIBCO BRL, Gaithersburg, MD). After tryptic digestion, the cells (passages 11-32) were washed once, separated from dead cells using ficoll-hypaque density gradient centrifugation, washed again, and then resuspended in tissue culture medium at 5 x 10⁶ cells/ml. Cell suspensions (400 µl) were added to the chambers of 12-mm nitrocellulose filters (Millipore-HA; Millipore Corp., Bedford, MA) and placed into 24-well tissue culture plates containing 400 µl culture medium.

MDM was replaced every other day until confluent monolayers with TEER of monolayers were measured using a TEER standard using a Multiple Regression computer program (ốiASSAY (12)).

Measurement of Transcytosis. The ability of Caco-2 cells to transcytose toxins was tested using confluent monolayers grown on nitrocellulose filters as described above. Media were removed from selected filters and 400 µl of fresh culture medium containing various concentrations of each toxin (SEA, SEB, TSSST-1) were added together to either the same apical (upper) or basal (lower) chamber of each culture. In each case an equal quantity of the enzyme horseradish peroxidase (HRP) was added as an internal control for nonspecific transcytosis due to leakage, fluid pinocytosis, etc. Among the available fluid phase markers, HRP (40 kD) was chosen because its molecular weight is close to those of the toxins (22-28 kD) so their pinocytosis should occur at more or less the same rate. Another 400 µl of culture medium was added to the other chamber. After incubation at 37°C for the indicated period of time, apical or basal media were collected depending on whether toxins were added to the basal or apical media. Amounts of each toxin and HRP present in collected samples were assayed as described below.

ELISA Analysis. A capture ELISA was used to determine amounts of each toxin present in different samples. For SEB, wells of Immulon® 3 plates (Dynatech Labs., Inc., Chantilly, VA) were coated overnight at 4°C with 100 µl of capture antibody B327 (10 µg/ml PBS), washed, and blocked with 25% FCS. Serial dilutions of the samples were added and the plates incubated overnight at 4°C. The captured toxin was detected using biotinylated B344 mAb followed by alkaline phosphatase-conjugated extravidin (Fisher Scientific, Pittsburgh, PA) and p-nitrophenyl phosphate as substrate. The binding sites on SEB of B327 and B344 do not overlap, nor does substitution of residue N23 or F44 significantly reduce their binding (23). The O.D. of the wells was read at 405 nm using an automated plate reader (Bio-Tek Instruments, Winooski, VT). Samples containing a known concentration of SEB were used to construct a standard curve. The amount of SEB present in each sample was calculated by comparison to the standard using a multiple regression computer program (őİASSAY (available on request). In the experiments where all toxins (SEA, SEB, and TSSST-1) and HRP were added to the same filter, specific sets of mAbs (described above) were used to determine the concentration of each toxin in the sample using essentially the same procedure.

An enzymatic assay was used to determine the concentrations of HRP in collected samples. In brief, samples were titered in PBS, and then 100 µl of TIMB microwell peroxidase substrate (Kirkegaard & Perry Labs., Inc., Gaithersburg, MD) was added to each well and incubated at room temperature until blue color developed. Wells were read at 630 nm using the ELISA reader. Concentration of HRP in each sample was determined using computer analysis by comparison to the HRP standard.

IL-2 Assay. Stimulation of T cell hybridomas to produce IL-2 was done in 96-well microtiter plates as previously described (27) using 5 x 10⁵ hybridoma cells and 5 x 10⁴ DR1-transfected fibroblast (DAP-DR1) as APCs of toxins. IL-2 production was used as a readout of T cell activation and was measured using the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (28).

Flow Cytometric Analysis. T cells purified from blood of mice treated with SEA or SEB were stained simultaneously with biotinylated anti-Vb and FITC-conjugated anti-CD4 antibodies and analyzed on a FACScan® (Becton Dickinson, San Jose, CA) as described elsewhere (12).

Oral Administration of Toxins to Mice. Toxins were administered to mice either orally or intraperitoneally. For oral administration of toxins, a special curved needle (Fisher Scientific) was used.
Soybean trypsin inhibitor (Sigma Chemical Co.) was added to reduce gastric digestion of orally administered toxins (29). Sera were collected from the treated mice and then tested for presence of toxins as described below.

Levels of functional toxins in sera. The ELISA described above was not sensitive enough to detect the low levels of toxins present in the blood of mice a few hours after administration of the toxins. Therefore, a functional assay was used which was at least one order of magnitude more sensitive than the ELISA. Dilutions of a serum to be tested for SEA or SEB were used in vitro to stimulate a T cell hybridoma bearing the appropriate Vβ element (Vβ3 for SEA and Vβ8.2 for SEB). At 24 h, the level of IL-2 produced was assayed as described above. As controls, dilutions of a standard preparation of SEA or SEB were tested similarly. A plot of the volume of sera or standard used versus the amount of IL-2 produced was constructed and the amount of toxin in the sera calculated by comparison to the SA antigen standard using multiple regression. Since the highest amount of serum that could be added to a culture was 10 μl, the limit of sensitivity of this assay was ~10 pg/ml for SEA and ~100 pg/ml for SEB.

**Results**

Transcytosis of SEB through Caco-2 Monolayers. We prepared two chambered cultures separated by a monolayer of confluent Caco-2 cells as described in Materials and Methods (see Fig. 1). We initially studied SEB transcytosis to characterize this system, using HRP as an internal control to distinguish between specific transcytosis and nonspecific mechanisms such as paracellular diffusion with water (30). Two types of experiments were done. In both, SEB and HRP were added to either apical or basal media and their appearance in the opposite chamber assessed as described in the Materials and Methods. In the first experiment (Fig. 2a), a fixed concentration of each protein (7.5 μg/ml) was used and the kinetics of transcytosis followed in either direction through the monolayer. In the second (Fig. 2b), various concentrations of the protein were added to the apical media and the amount transcytosed to the basal medium assayed at 90 min. These results established several points. First, transcytosis of SEB was detected in both directions across the Caco-2 monolayer and the rate of SEB transcytosis was linear for the 24 h of the experiment. Second, SEB was transcytosed faster than HRP, especially in the apical to basolateral direction where the SEB transcytosis rate was usually three to six times faster than that of HRP. These results suggested that the transcytosis of SEB by Caco-2 was facilitated, perhaps by a specific surface receptor. However, we found no evidence in these experiments for saturation of the SEB transcytosis mechanism even using concentrations of SEB up to 300 μg/ml (Fig. 2b).

Facilitated transcytosis of SEB and TSST-1 but not SEA by Caco-2 Cells. To see if this accelerated transcytosis was specific to SEB, we tested two other staphylococcal exotoxins in this system, SEA and TSST-1. All three toxins were added to either the basal or apical medium of Caco-2 cultures and their appearance in the opposite chamber followed. Again, HRP was included in each culture as internal control. The results (Fig. 3) showed that TSST-1 was transcytosed by Caco-2 cells similarly to SEB. In each case, transcytosis was bidirectional and severalfold faster than the rate of HRP transcytosis. Also in both cases we again saw no evidence for saturation of the transcytosis mechanism with the concentration of toxins used here (up to 4 μg/ml). The results with SEA were very different. Although SEA was transcytosed in both directions, the rate was very similar to that of HRP, suggesting that SEA crossed the monolayer nonspecifically with water.

**Figure 2.** (a) SEB transcytosis by Caco-2 cells is bidirectional and time dependent. (Apical to basal) Toxin and HRP (3 μg each in 400 μl medium) were added to the apical chamber. Samples were collected from basal chambers at indicated time points over a period of 24 h. Transcytosed toxin and HRP were then measured as described in Materials and Methods. (Basal to apical) The experiment was done as above except the toxin and HRP were added to the basal chamber and apical samples were collected. Each point is the mean of three determinants. SEM was usually <15%. This experiment is a representative of three similar experiments. (b) Transcytosis of SEB by Caco-2 cells is dose dependent but not saturable. Different concentrations of SEB and HRP were added to the apical medium of the same filter. Basal media were collected 90 min later and assayed for SEB and HRP transcytosed. Each point was performed in triplicate. SEM was usually <15%. This experiment is a representative of three separate experiments with similar results.
stimulate T cells by at least 1,000-fold (22). Either mutation reduced the rate of SEB transcytosis by 50–70% (Fig. 4). These results suggest that these regions of SEB have a function in the transcytosis and provide additional evidence for the specificity of SEB transcytosis by Caco-2 cells.

Presentation of SEB but Not SEA to T Cells by Caco-2. Our results suggested that transcytosis by Caco-2 cells of SEB, but not SEA, may be facilitated by a specific receptor. To examine this idea further, we tested the ability of Caco-2 cells to present SEA and SEB to T cell hybridomas bearing the appropriate Vβ regions in their T cell receptors. This experiment was possible because, unlike normal T cells, T cell hybridomas do not require professional APC-specific co-receptors for activation. Also, recent experiments have established that MHC class II is not an essential part of the SAγ ligand for T cells, provided that the SAγ is presented in the proper orientation for T cell receptor interaction (23, 33–35).

We cultured a Vβ3 and Vβ8.2 T cell hybridoma with various concentrations of SEA or SEB in the presence of either Caco-2 or DR1+ fibroblasts as the APCs. The results are shown in Fig. 5. The DR1-bearing fibroblast presented both SEA and SEB efficiently to the appropriate T cell hybridoma. On the other hand, Caco-2 presented SEB, but not SEA. However, Caco-2 was about 100-fold less efficient than the DR1+ fibroblast in presenting SEB, possibly indicating a very low affinity receptor.

Transcytosis of SEA and SEB in Mice. Transcytosis of SEB and TSST-1 by Caco-2 cells shows that intestinal epithelia have the potential to deliver toxins across the intestine. To study transcytosis under more physiological conditions, we tested the ability of orally administered toxins to cross the intestinal barrier of mice and enter systemic circulation without altering their functional capacity. Since TSST-1 is labile to digestion by proteolytic enzymes of the gastrointestinal tract especially pepsin (36), we limited the in vivo experiments to SEA and SEB which are fairly resistant to digestion by gastrointestinal secretions (37). SEA or SEB were introduced into the stomachs of mice and the appearance of the functional toxin in the circulation was followed either by using serum from these mice to stimulate in vitro T cell hybridomas bearing the appropriate Vβ elements or by following the in vivo deletion of T cells bearing these Vβs over the course of several weeks.

Results from the in vitro stimulation experiments are shown in Fig. 6. Various quantities of sera taken from mice after feeding either SEA or SEB were assessed for their ability to stimulate IL-2 production by Vβ3- or Vβ8.2-bearing T cell hybridomas, respectively, using DR1+ APCs. Preliminary experiments established that serum levels of toxins peaked at about 2 h (data not shown). Typical titrations are shown in Fig. 6 a. These titration data were used to calculate the levels of functional toxins in the sera as described in Materials and Methods. These data are summarized for all mice in Fig. 6 b. Functional SEB and SEA were easily detected in the sera of most of the mice. There was considerable variation in the levels achieved, particularly of SEB.

Figure 3. Transcytosis of SEB and TSST-1, but not SEA, by Caco-2 cells. (A) Apical to basal. Equal concentrations of each toxin and HRP were added to the apical media of the same filter. Basal media were collected and assayed for toxins and HRP as indicated in Materials and Methods (B) Basal to apical. The same experiment done in the opposite direction. Each point was performed in triplicate. SEM was usually <15%. This experiment is representative of at least four separate experiments with similar results.

Figure 4. Mutation of residues N23 and F44 affects SEB transcytosis by Caco-2 cells. (A) The amount of wild type and mutants SEB transcytosed (apical to basal) per filter in 4 h. The experiment was done as in Fig. 3. Wild type and mutant SEB were used to construct standard curves. The amounts of wild type and mutants transcytosed relative to that of HRP. Each point is the mean of three determinations. SEM was usually <15%. This experiment is representative of three separate experiments with similar results.

Figure 5. Presentation of SEB, but not SEA, by Caco-2 cells. (A) Monolayers of Caco-2 cells (B) were used to present various concentrations of each toxin to specific T cell hybridomas. SEA reactive (Vβ3+) and SEB reactive (Vβ8.2+) T cell hybrids were used. (B) As a positive control, DR1+ LG2 cells were used as APCs. IL-2 produced by activated T cells was measured as described in the Materials and Methods. This experiment is representative of four similar experiments.

Figure 6. Presentation of SEA and SEB to T cells by Caco-2. (A) A positive control, DR1+ LG2 cells were used as APCs. IL-2 produced by activated T cells was measured as described in the Materials and Methods. This experiment is representative of four similar experiments.
but on average SEB reached levels (2,750 pg/ml) more than 20 times those of SEA (100 pg/ml). This difference may be related to the facilitated transcytosis of SEB versus the apparently passive transcytosis of SEA seen in vitro with human Caco-2 cells. However, other explanations such as differential degradation or clearance of the two toxins in vivo cannot be excluded by our data.

As a second indication of the transcytosis of functional SEA and SEB in these mice, we followed in vivo the fate of VB3- and VB8-bearing T cells. Exposure of mice to systemic injections of SAgs usually leads to expansion of T cells (maximum around day 2) bearing the appropriate VBs followed by clonal deletion that becomes evident between 7 and 10 d after injection (38). Therefore, we examined the blood of the mice for the levels of T cells bearing these VBs on day 2 and 10 after feeding SEA or SEB, and compared these results to those obtained with an intraperitoneal injection of the same dose of toxins. The results are shown in Fig. 7. In both cases, as expected, intraperitoneal injection led to expansion of the appropriate T cell pool on day 2 followed by substantial deletion by day 10. Feeding the same amount of toxin, however, led to substantial deletion on day 10 that was not preceded by an expansion on day 2. The most likely explanation for this difference lies in the lower effective level of toxin achieved with feeding over direct injection, since direct injection of very low doses of toxin has been shown to lead to in vivo T cell deletion that is not preceded by detectable T cell expansion (38). Taken together, these results show that SEB and SEA readily cross the intestinal epithelium and enter the blood stream in a fully functional form.

Discussion

The main conclusion of these studies is that the staphylococcal exotoxins, SEB, SEA, and TSST-1, can rapidly cross an epithelial membrane in intact, fully functional forms. These findings have implications for the role of SEB, SEA, and similar SAgs enterotoxins in food poisoning since, if the SAg activity of these toxins is important in this pathology, they must have the ability to cross the intestinal epithelium intact to gain access to T cells.

Our experiments with the human polarized enterocytic cell line Caco-2, suggest that these toxins may cross intestinal epithelium by more than one mechanism. The rate of SEA transcytosis was similar to that of a marker for water transport, HRP, suggesting a nonspecific mechanism. SEB, on the other hand, was transcytosed at three to six times the rate of HRP, suggesting a receptor-facilitated process. Correlating with this idea was the ability of Caco-2 to act as an APC for SEB, but not SEA, in stimulating T cell hy-
bridomas bearing the appropriate Vβ element. Other evidence in support of this conclusion was the inhibition of SEB transcytosis by mutations in N23 or F44. These mutations also interfere with the SAg activity of SEB by disrupting T cell receptor and MHC class II binding, respectively. Previously, Harris et al. (37) has shown that these mutations also interfere with SEB-induced food poisoning in monkeys. While interpreted at the time in terms of the SAg activity of SEB, our current findings suggest that the reduction in SEB transcytosis caused by these mutations may have contributed as well to the results.

Despite the appearance of a receptor-mediated mechanism for SEB transcytosis, several features usually associated with such a mechanism were not observed. The rate of transcytosis was only slightly favored in the apical to basal direction through the Caco-2 monolayer. Usually, transcytosis is biased in one direction because the distribution of the receptor involved is polarized. For example, in intestinal and mammary epithelium, transcytosis of secretory IgA from the basal to apical surface occurs via an Fc receptor (plgR) whose distribution is polarized in favor of the basolateral surface (39–43). Reciprocally, absorption of IgG from ingested milk in neonatal rats and mice is mediated by a specific Fc receptor (FcRn), and polarized to the apical surface of the intestinal epithelium (44, 45). However, there are, in fact, examples of bidirectional transcytosis of ligands such as glutathione (46) and glycylysarcosine (47). Also, there is a recent description by Ellis and Luzio (48) of a novel receptor on Caco-2 cells that can transcytose in both directions.

More importantly, in our experiments we saw no evidence for saturation of SEB transcytosis, even using SEB concentrations up to 300 μg/ml. If this were a receptor-mediated process, we would have expected the rate to maximize as the receptor became saturated (at concentrations of ~10 times the kD). These results suggest that the putative receptor must be of low affinity (~kD > 5 × 10<sup>-5</sup> M<sup>-1</sup>), a result consistent with the finding that SEB presentation to T cells by Caco-2 is much less efficient than by MHC class II<sup>+</sup> fibroblast. (The MHC class II affinity for SEB is ~10<sup>-6</sup> M<sup>-1</sup>.)

Surprisingly, we observed facilitated transcytosis of TSST-1 as well as SEB by Caco-2 cells. Again, this transcytosis occurred at a rate about fivefold greater than that of HRP and was bidirectional and could not be saturated. TSST-1 is not an enterotoxin, most likely because it is destroyed by digestive enzymes in the stomach and intestine (49). However, TSST-1 is a major cause of toxic shock syndrome. In women, TSST-1 produced in the vagina can find its way into the blood stream, where the subsequent toxic shock is accompanied by massive stimulation of peripheral Vβ2-bearing T cells (13, 15). Since these cases are often associated with use of a particular type of tampon during menses, the toxin may enter the body via direct contact with blood. However, if the putative receptor mediating TSST-1 transcytosis through Caco-2 is common to other types of epithelium, then transcytosis may contribute as well to the uptake of the toxim. This notion is supported by the ability of conjunctival epithelia and endothelia to endocytose and transcytose TSST-1 (50).

Previous studies have shown that there are non-MHC receptors for some of the staphylococcal toxins on intestinal epithelium, B cells, and mast cells (33–35). These studies showed that human intestinal epithelial cells and mouse B cells or mast cells could present SEB, but not SEA. In contrast, lymph node cells from class II<sup>-</sup>-deficient mice could present SEC1-3 and SEE, but not SEB or SEA. Some of these results are similar to those described in this paper. Perhaps, the receptors which participate in toxins binding and transcytosis are related to some of these reported molecules.

In an attempt to relate our results to a more physiological situation we documented the appearance of ingested SEA and SEB in the blood of mice. Functional toxin was detected both by the ability of serum from these animal to stimulate the appropriate T cell hybridomas in vitro and by the in vivo partial disappearance of T cells bearing the appropriate Vβ elements. These results extend those of Migita and Ochi (29) who used Western blotting to show the appearance of intact SEB in the blood of mice fed the toxin. As in our in vitro transcytosis studies, we found that the extent of SEA uptake was less than that of SEB, although both rapidly reached functional levels in the serum. This could again reflect a passive versus active mechanism of transcytosis, but other mechanisms, such as differential degradation, are not excluded by our results.

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