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FINAL REPORT

Grant Number: DAAG 29-78-G-0087

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Cincinnati, Ohio 45267

Title: Membrane associated phenomena associated with Staphylococcal
enterotoxins.

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Statement of problem studied:

- a) To determine if SE's exert any cytotoxic effects on a human intestinal epithelial cell line in vitro;
- b) To demonstrate specific toxin binding to murine lymphocytes via specific toxin receptors on the cell membrane.

Summary of experimental results: see following.

SUMMARY (#DAAG 29-78-G-0087)

The interaction of staphylococcal enterotoxin A (SEA) with mammalian cell membranes was studied in two in vitro systems. The cytotoxic properties of SEA were evaluated using the Henle 407 human embryonic intestinal epithelial cell line (Henle 407). The specific binding of ^{125}I -SEA was determined for mouse lymphoid cells.

The effects of staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB) on mammalian cell integrity and function as measured by leakage of labelled cytoplasmic constituents, amino acid transport, and macromolecular synthesis, was evaluated for Henle 407 cells. No evidence of cytotoxicity by any of these criteria could be detected for cell monolayers incubated with SEA for periods between 30 min and 24 hr. Purified staphylococcal hemolysins (alpha toxin and delta toxin) were shown to exert cytotoxicity by the amino acid leakage uptake assays. In efforts to detect synergistic effects between enterotoxin and the staphylococcal cytotoxins, membrane functions were evaluated after sequential or combined treatment with enterotoxin and alpha toxin, or enterotoxin and delta toxin. In no instance could a contribution to cytotoxicity by the staphylococcal enterotoxin be detected. That the assays were sufficiently sensitive to detect synergistic effects was shown by the greater than additive effects achieved with a combination of alpha and delta toxins. The data show that staphylococcal enterotoxins do not behave as bacterial cytotoxins.

The activity of SEA as a T-lymphocyte mitogen was used in studies of the initial interaction between mouse lymphoid cell membranes and the enterotoxin. An assay was developed to evaluate the specific binding of ^{125}I -labelled SEA (^{125}I -SEA) to mouse lymphoid cell receptors. Binding was specific in that it was inhibited by unlabelled SEA but not by unrelated proteins. The amount of specific binding correlated with the amount of mitogenic stimulation. Specific binding increased rapidly with time to a steady state and increased to saturation at high concentrations of ^{125}I -SEA. The amount of specific binding was also sensitive to temperature and pH. Scatchard analysis indicated a single class of binding sites with an equilibrium dissociation

constant of $8 \times 10^{-7} \text{M}$ and approximately 3600 binding sites per spleen cell. In addition to SEA and antigenically distinct unlabelled staphylococcal enterotoxins, staphylococcal enterotoxin B and staphylococcal enterotoxin E, competitively inhibited binding of ^{125}I -SEA to mouse lymphoid cells. This indicates a common class of binding sites for these three staphylococcal enterotoxins.

Evaluation of ^{125}I -SEA specific binding sites on mouse lymphoid cell subpopulations showed that T-lymphocytes bind less ^{125}I -SEA than do B-lymphocytes. ^{125}I -SEA did not bind to macrophages in detectable amounts. However, an evaluation of the cell-cell interactions involved in the mitogenic response to SEA showed that both B-lymphocytes and macrophages participate in the T-lymphocyte mitogenic response to the enterotoxin. Splenic B-lymphocytes greatly enhanced the otherwise weak mitogenic response of thymic T-lymphocytes to SEA. Also, macrophages apparently take up SEA by a pinocytic process which is temperature and cyanide sensitive. Thus, macrophages exposed to SEA for 3 hr, then thoroughly washed, trigger mitogenesis in fresh spleen cell cultures without additional SEA. The specific interaction between SEA and lymphoid cells and the corresponding lack of demonstrable direct cytotoxicity for SEA supports an hypothesis that SEA may act in vivo via an immunologic rather than a direct cytotoxic mechanism.

Another alternative which was explored in a preliminary way is that SE's may act on nerve cells. This would be compatible with the notion espoused by several investigators in the past that SE's exert their emetic action by direct effects on the nervous system. We tested SEA and SEB on a neuroblastoma cell line and found that the toxins induced neurite formation in vitro. This "differentiation" of the neuroblastoma cell upon exposure to the enterotoxins indicates a direct cell-cell interaction; i.e. that toxin interacts with the nerve cell by a specific toxin ligand-cell receptor mechanism. This exciting observation will form the basis for a new research grant application to be submitted to the Army Research Office later this year.

List of Publications:

- *1. Staphylococcal enterotoxins fail to disrupt membrane integrity or synthetic functions of Henle 407 intestinal cells. 1981. Infection and Immunity, 31:929-934. (S. Buxser and P.F. Bonventre).
- **2. Specific receptor binding of Staphylococcal enterotoxins by murine splenic lymphocytes. 1981. Infection and Immunity (in press; September 1981). (S. Buxser, P.F. Bonventre, and D. Archer).
- ***3. Cell-cell interactions in the mitogenic response to staphylococcal enterotoxins. (in preparation for Immunopharmacology). (S. Buxser, D. Archer, and P.F. Bonventre).

* Is published; reprints enclosed as part of the final report.

** Is accepted for publication; copies of manuscript enclosed as part of the final report.

*** Has not yet been accepted; principal observations are included in the summary of the final report and reprints will be forwarded when they become available.

Advanced degrees: Steven Buxser was supported by the ARO grant and was awarded the Ph.D. degree from the University of Cincinnati in 1981.

Staphylococcal Enterotoxins Fail to Disrupt Membrane Integrity or Synthetic Functions of Henle 407 Intestinal Cells

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The potential cytotoxic activity of purified staphylococcal enterotoxins for mammalian cells was evaluated. The effects of staphylococcal enterotoxins A (SEA) and B (SEB) on cell membrane integrity as measured by leakage of labeled cytoplasmic constituents ($[^3\text{H}]$ uridine), amino acid transport (lysine and amino-isobutyric acid), and macromolecular synthesis (protein, ribonucleic acid, and deoxyribonucleic acid) was evaluated for a human intestinal epithelial cell (Henle 407). No evidence of cytotoxicity by any of these criteria could be detected for cell monolayers incubated with SEA for periods of between 30 min and 24 h. Purified staphylococcal hemolysins (alpha- and delta-toxins) were shown to exert cytotoxicity by the leakage and amino acid uptake assays. In efforts to detect synergistic effects between enterotoxin and the staphylococcal cytotoxins, membrane functions were evaluated after sequential or combined treatment with enterotoxin and alpha-toxin or with enterotoxin and delta-toxin. In no instance could a contribution to cytotoxicity by the staphylococcal enterotoxin be detected. That the assays were sufficiently sensitive to detect synergistic effects was shown by the greater than additive effects achieved with a combination of alpha- and delta-toxins. The data, contrary to previous reports, showed that staphylococcal enterotoxins did not behave as bacterial cytotoxins.

Several antigenically distinct staphylococcal enterotoxins are produced by a significant percentage of *Staphylococcus aureus* clinical isolates. The toxins cause severe food poisoning in humans. Symptoms of this clinical entity include vomiting, diarrhea, and nausea, usually within 8 h after ingestion (2), and the toxin serotypes produce the same clinical syndrome. Although enterotoxins A (SEA), B (SEB), and C are distinct proteins, they have similar physiochemical properties (1). SEA is most frequently associated with human food poisoning outbreaks. Furthermore, this toxin serotype is most readily available in highly purified form (18). Therefore, SEA was chosen for this study, although some experiments using SEB were also included.

The biochemical mechanism by which staphylococcal enterotoxin acts is unknown. One of several mechanisms suggested is a direct cytotoxic effect of ingested toxin on gastrointestinal tissues. Observations that support this view include acute exogenous gastritis observed in outbreaks of staphylococcal food poisoning (11), lesions of the small intestine observed in rhesus monkeys given SEB by mouth (8), and changes in intestinal transport of fluids and electrolytes as a result of the administration of enterotoxins (6, 19). These data suggest that staphylococcal enterotoxins may disrupt normal intestinal physiology but do not describe what changes, if

any, occur in intestinal mucosal cells. Opportunity for study of toxin action is provided by in vitro experimental models.

Using an established tissue culture line of embryonic intestinal mucosa (Henle 407), Schaffer et al. (15, 16) and Schaffer (14) observed a cytopathic effect and a significant reduction in cellular protein of monolayers treated with enterotoxin for periods of between 24 and 48 h. Although microscopically visible cytopathic changes and apparent cessation of cell growth suggested a direct cytotoxic action of enterotoxin on intestinal epithelial cells, these observations were not conclusive. There have been no subsequent studies on the putative cytotoxic action of staphylococcal enterotoxins by using sensitive and quantitative assays currently available to detect mammalian cell damage. The in vitro assay developed by Thelestam et al. (20-22), which quantitatively measures leakage of low-molecular-weight compounds from previously labeled mammalian cells, and an assay developed by Duncan and Buckingham (3), which measures inhibition in amino acid or sugar uptake as a result of subtle membrane damage, are extremely sensitive indicators by which cytotoxicity may be assessed. Thus, these assays were used to evaluate cell damage induced by SEA and SEB. In addition, we also measured several metabolic parameters of macromolecular syn-

thesis in efforts to detect changes produced as a result of enterotoxin interaction with the cells.

Since no definitive evidence exists that staphylococcal enterotoxins are in fact cytotoxins, we included as positive controls two staphylococcal toxins which are recognized cytotoxic agents. In addition, these staphylococcal toxins were used in experiments in which treatment of mammalian cells with either alpha- or delta-toxin was combined either simultaneously or sequentially with staphylococcal enterotoxin. The rationale for such experiments was to detect effects of enterotoxin which might not be detected by treatment with the enterotoxin alone. A synergistic effect on cells by more than a single toxin of staphylococcal origin was a possibility which required testing.

The data which were obtained, however, showed unequivocally that SEA and SEB did not behave as cytotoxic protein toxins on the Henle 407 cell line. Enterotoxin caused no alteration in membrane integrity or function, and cellular macromolecular synthesis was unaffected by treatment with toxin at concentrations exceeding by several orders of magnitude those required for induction of emesis in humans (1).

MATERIALS AND METHODS

Isotopes. Radioisotopes were obtained from New England Nuclear Corp., Boston, Mass.: [α -methyl- 3 H]aminoisobutyric acid (AIB; specific activity, 10 Ci/mmol); [α - 14 C]AIB (specific activity, 51.6 Ci/mmol); L-[3 H]lysine (specific activity, 4.1 Ci/mmol); and [5,6- 3 H]uridine (specific activity, 40.8 Ci/mmol).

Toxins. Staphylococcal alpha-toxin was obtained from Sidney Harshman, Vanderbilt University, Nashville, Tenn. Purified toxin contained 16,000 hemolytic units per mg of protein. Alan Bernheimer, New York University School of Medicine, kindly supplied purified delta-toxin. The delta-toxin preparation contained 100 hemolytic units per mg of protein. SEA and SEB were provided by Leonard Spero, U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Md., and Merlin Bergdoll, Food Research Institute, University of Wisconsin, Madison. The enterotoxins had been purified by the method of Scharitz et al. (17, 18). The enterotoxin preparations were free of contaminating hemolysins.

Tissue culture and tissue culture medium. The Henle 407 human intestinal epithelial cell line (ATCC CCL6) was obtained from the American Type Culture Collection, Rockville, Md. This cell line was established originally by W. Henle from jejunum and ileum of a 2-month-old human embryo. 10T1/2 fibroblasts of C3H mouse origin were obtained from George Todaro, National Cancer Institute, Bethesda, Md. Tissue culture medium was obtained from GIBCO Laboratories, Grand Island, N.Y. Growth medium consisted of Hanks balanced salts solution (HBSS), basal Eagle medium (BME), and 10% fetal calf serum. Tissue culture plastic supplies were obtained from the Costar Division of Bellco Glass, Vineland, N.J., both 6 well

(no. 3506) and 24-well (no. 3524) cluster dishes were used.

[3 H]uridine leakage assay. The assays described originally by Thelestam et al. (20-22) were used to detect cytotoxicity. Henle 407 human epithelial cells and 10T1/2 mouse fibroblasts were cultured as monolayers in 24-well tissue culture plates in BME plus 10% fetal calf serum as the growth medium. Cells were grown to less than total confluence before use in experiments. [3 H]uridine labeling of cells was accomplished by removing growth medium from the monolayers and replacing it with fresh BME containing 10 μ Ci of [3 H]uridine per ml for 2 h followed by a 2-h chase with medium free of radiolabel. The cells were washed three times with tris(hydroxymethyl)aminomethane-buffered saline (pH 7.5) followed by incubation with or without toxin in the buffer for 30 min. The supernatant was aspirated, and radioactivity in a 0.1-ml portion in 10 ml of Thrift Solve (Kew Scientific, Columbus, Ohio) was counted to determine the quantity of uridine which leaked from the cells. Maximal release of cellular label was determined by disrupting the cells with 0.1% Triton X-100.

The amount of [3 H]uridine released from the cells as a consequence of specific membrane damage was calculated according to the formula.

% release

$$= \frac{\text{cpm released} - \text{cpm spontaneously released}}{\text{total cpm} - \text{cpm spontaneously released}} \times 100$$

Amino acid uptake assay. Duncan and Buckingham (3) showed that inhibition of hexose or amino acid uptake was a more sensitive indicator of membrane damage than assays based on leakage of intracellular components. Henle 407 cells were grown to near confluence in six-well (35-mm diameter) plastic tissue culture dishes. BME was aspirated, and each well was rinsed two times with HBSS. The plates were floated on a water bath at 37°C, and 2 ml of [14 C]AIB (0.5 μ Ci/ml in 1.0 mM AIB) or L-[3 H]lysine (1 μ Ci/ml in 1.0 mM L-lysine) in HBSS was added to the monolayers in each well for 20 min. Preliminary experiments indicated that the uptake of both amino acids had attained saturation within this 20-min period. At the conclusion of incubation, the labeled amino acid solution was aspirated, the dish was placed on ice, and each well was rinsed quickly three times with 3 ml of cold phosphate-buffered saline (PBS, pH 7.4). Cells were solubilized in 1.5 ml of 0.2 N NaOH, and 0.5 ml was removed for protein determination (10). A 1.0-ml sample neutralized with HCl was added to a scintillation vial containing 12 ml of Kewsolve (Kew Scientific) and counted in a Packard model 2125 scintillation spectrophotometer.

Several experiments involved use of two amino acids simultaneously, in which case both [14 C]AIB at 0.5 μ Ci/ml and L-[3 H]lysine at 1.0 μ Ci/ml were added to the incubation medium. The window settings for discriminating 14 C and 3 H in double-labeling experiments were determined as described by Beckman Instruments, Inc. (technical report 915 NUC-76-77).

SEA effects on cells. SEA was dissolved in PBS at 200 times the desired final concentration. At the beginning of each experiment, a 10 μ l portion of SEA

was added to each well containing 2 ml of tissue culture medium and mixed well. Cells were incubated at 37°C for the indicated period in a 5% CO₂ atmosphere before rinsing in HBSS and processing for evaluation of amino acid transport as described above.

Macromolecular synthesis by cells treated with SEA. Henle 407 cells grown for 3 days in 24-well tissue culture dishes were used for ascertaining de novo protein, ribonucleic acid (RNA), or deoxyribonucleic acid (DNA) synthesis. Spent medium was aspirated, and fresh medium containing SEA or a recognized inhibitor of macromolecular synthesis as a positive control was added to the wells. *Pseudomonas* exotoxin A was used as an inhibitor of protein synthesis (12), actinomycin D was used as an inhibitor of RNA synthesis, and mitomycin C was used as an inhibitor of DNA synthesis. To determine protein synthesis, [³H]leucine (2 µCi/ml) was added for 90 min; RNA synthesis was determined after 24 h of incubation with [³H]uridine at a concentration of 1 µCi/ml; and DNA synthesis was determined after 4 h of incubation with 1 µCi of [³H]thymidine per ml. All radiolabeled solutions were added in fresh BME with 10% fetal calf serum which also contained the appropriate radiolabel and toxin at the concentration noted in Table 6. Cells in each well were dissolved in 0.1% sodium dodecyl sulfate and precipitated with an equal volume of 10% trichloroacetic acid. The resulting precipitate was washed with 5% trichloroacetic acid, dissolved in 1 N NaOH, and evaluated by liquid scintillation counting.

Statistical analysis. Statistical analysis for experiments with equal numbers of replicates was done by using analysis of variance as described by Hicks (5). Analysis of data with unequal replicates was done by using the General Linear Models program of the Statistical Analysis System computer package at the University of Cincinnati Computer Center. As described by Hicks (5), the "interaction" term referred to in Tables 2 and 3 is a measure of the change in response of one factor under the influence of a second factor. Specifically, a statistically significant interaction was interpreted as cell damage due to a combined toxin treatment greater than the sum of effects of the toxins administered separately, i.e., a synergistic effect. For data statistically significant as measured by analysis of variance or the general linear model, the means which were statistically significant were determined by Duncan's multiple-range test.

RESULTS

Synergistic action of staphylococcal alpha- and delta-toxins. The effects of staphylococcal alpha-toxin, delta-toxin, SEA, and SEB on leakage of [³H]uridine from prelabeled cells are shown in Table 1. Only delta-toxin was overtly cytotoxic as measured by increased leakage of [³H]uridine by 10T1/2 fibroblasts or Henle 407 cells. A similar leakage assay (data not shown) using cells prelabeled with [³H]-AIB detected damage induced by staphylococcal alpha-toxin as well as the delta-toxin. However, with either assay, no leakage of uridine or AIB

TABLE 1. [³H]uridine leakage from prelabeled cells exposed to staphylococcal toxins^a

Cell type	Toxin (µg/ml)	Uridine leakage (% maximum)
10T1/2 mouse fibroblast	Enterotoxin A (10)	2.8 ± 2.0
	Enterotoxin A (20)	-0.1 ± 1.6
	Enterotoxin A (100)	4.4 ± 1.5
	Enterotoxin B (100)	3.0 ± 2.3
	Delta-toxin (1.0)	3.0 ± 1.2
	Delta-toxin (2.5)	8.8 ± 6.6 ^b
	Delta-toxin (5.0)	18.8 ± 5.0 ^b
	Delta-toxin (25.0)	64.4 ± 14.4 ^b
	Alpha-toxin (3.2)	5.5 ± 3.5
Henle 407 human intestinal epithelial	Enterotoxin A (10)	1.8 ± 1.3
	Enterotoxin A (20)	2.7 ± 0.6
	Enterotoxin A (100)	2.8 ± 1.5
	Enterotoxin B (100)	4.2 ± 1.8
	Delta-toxin (1.0)	2.8 ± 1.1
	Delta-toxin (2.5)	0.2 ± 3.2
	Delta-toxin (5.0)	3.8 ± 4.5
	Delta-toxin (25.0)	19.9 ± 12.1 ^b
	Alpha-toxin (0.3)	2.2 ± 1.6
	Alpha-toxin (3.2)	3.0 ± 1.4

^a Each value is the mean of 6 to 12 determinations ± standard deviation. Henle 407 cells or 10T1/2 mouse fibroblasts were grown to near confluence on 24-well tissue culture plates. Spent medium was aspirated and replaced with fresh medium containing 1 µCi of [³H]uridine per ml. A 2-h incubation was followed by a 2-h chase with fresh medium without radiolabeled components. The cells were washed three times with tris(hydroxymethyl)aminomethane-buffered saline followed by incubation with toxin in the buffer for 30 min at 37°C. Sephadex G-25 gel chromatography revealed that cells labeled by this technique leaked materials of low molecular weight (<2,500) that therefore represented free uridine and oligonucleotides.

^b Statistically significant (*P* < 0.05).

greater than control values was induced by SEA.

The assay of Buckingham and Duncan (3), which measures uptake of amino acids by tissue culture cells after toxin treatment, was also used in efforts to detect cytotoxicity. The amino acids lysine and AIB were chosen for study since they represent amino acid species taken into mammalian cells via different transport systems (4). Although independent transport systems were not demonstrated specifically for Henle 407 cells, our experiments showed that cold L-lysine in excess did not inhibit uptake of labeled AIB significantly, and, conversely, unlabeled AIB inhibited lysine uptake only slightly (data not shown). Thus, the two transport systems appeared to be independent in Henle 407 cells.

It was also important to evaluate the potential cytotoxicity of SEA and SEB together with the known cytotoxins staphylococcal alpha- and

delta-toxins. Therefore, as positive controls in the assays, the effects on AIB and lysine transport after treatment of Henle 407 cells with alpha- or delta-toxin alone or both toxins in combination are shown in Table 2. Delta-toxin did not substantially reduce transport of either AIB or lysine except at the highest dose tested (10 $\mu\text{g}/\text{ml}$). Alpha-toxin treatment of cells reduced transport of both amino acids at concentrations of 3 $\mu\text{g}/\text{ml}$ or greater. Combinations of alpha- and delta-toxins produced significant decreases in amino acid uptake at concentrations which, when administered singly, had no measurable inhibitory effect. For example, alpha- or delta-toxin at 1 $\mu\text{g}/\text{ml}$ was not inhibitory for amino acid transport. However, combined treatment with both toxins, each at that concentration, substantially reduced uptake of AIB (30%) and lysine (53%). Similar synergistic effects by alpha- and delta-toxins were evident at concen-

trations of 1 μg of alpha-toxin and 5 μg of delta-toxin per ml or 0.5 μg of alpha-toxin and 5 μg of delta-toxin per ml. The interaction of the effects of the alpha- and delta-toxins was statistically significant ($P < 0.0001$). These observations demonstrated the extreme sensitivity of the uptake assay for detection of subtle cell membrane perturbation and also showed that the assay could detect a synergistic action of two cytotoxic agents used at levels below threshold.

Absence of cytotoxicity of staphylococcal enterotoxin. The effects of a 24-h incubation with SEA at concentrations up to 10 $\mu\text{g}/\text{ml}$ on uptake of AIB and lysine are shown in Table 3. Enterotoxin alone had no significant effect on AIB uptake at the doses tested. Cells treated with enterotoxin for 24 h together with one of the hemolytic toxins for the last 30-min period showed decreases in transport of AIB and lysine which could be accounted for solely by the ac-

TABLE 2. Effect of combined staphylococcal hemolytic toxin treatment on amino acid uptake by Henle 407 cells^a

Alpha-toxin ($\mu\text{g}/\text{ml}$)	Amino acid uptake (% of control) with delta-toxin at							
	None		1.0 $\mu\text{g}/\text{ml}$		5.0 $\mu\text{g}/\text{ml}$		10.0 $\mu\text{g}/\text{ml}$	
	AIB	Lysine	AIB	Lysine	AIB	Lysine	AIB	Lysine
None	100.0 $\pm$ 3.4 ^b	100.0 $\pm$ 3.2 ^b	89.3 $\pm$ 2.5	101.2 $\pm$ 3.0	107.9 $\pm$ 10.5	104.2 $\pm$ 6.0	81.1 $\pm$ 5.9	89.3 $\pm$ 8.3
0.5	94.6 $\pm$ 4.6	99.9 $\pm$ 4.2	80.3 $\pm$ 4.1	94.4 $\pm$ 5.0	66.9 $\pm$ 2.0	80.9 $\pm$ 2.2	74.5 $\pm$ 6.1	82.9 $\pm$ 8.0
1.0	89.0 $\pm$ 5.7	104.0 $\pm$ 13.2	30.3 $\pm$ 3.9	53.4 $\pm$ 4.6	14.4 $\pm$ 0.8	34.5 $\pm$ 0.8	73.5 $\pm$ 12.7	Not done
3.0	38.1 $\pm$ 5.4	52.0 $\pm$ 8.4	36.2 $\pm$ 8.6	55.3 $\pm$ 10.1	15.0 $\pm$ 2.8	36.8 $\pm$ 3.5	11.0 $\pm$ 0.7	32.9 $\pm$ 0.4

^a Nearly confluent monolayers of Henle 407 cells in 35-mm wells were rinsed twice with HBSS. Toxin(s) at the concentrations noted was added in 2 ml of HBSS for 30 min and aspirated; then 2 ml of labeled amino acid solution containing 1 mM AIB (1 $\mu\text{Ci}/\text{ml}$) and 1 mM lysine (1 $\mu\text{Ci}/\text{ml}$) was added for a 20-min incubation at 37°C. Wells were rapidly washed three times with ice-cold PBS, and cells were dissolved in 1 ml of 0.1 N NaOH. Statistical analysis showed significant interaction of the combined alpha- and delta-toxin treatment on AIB uptake ($P < 0.0001$) and lysine uptake ($P = 0.0003$). The values listed are the means of 3 to 12 determinations $\pm$ standard deviation.

^b Normal control values for AIB and lysine uptake.

TABLE 3. Effect of prolonged SEA pretreatment of Henle 407 cells on amino acid uptake^a

Hemolytic toxin ($\mu\text{g}/\text{ml}$)	Amino acid uptake (% of control) with SEA at					
	None		1.0 $\mu\text{g}/\text{ml}$		10.0 $\mu\text{g}/\text{ml}$	
	AIB	Lysine	AIB	Lysine	AIB	Lysine
None	100.0 $\pm$ 2.1	100.0 $\pm$ 1.8	94.2 $\pm$ 4.6	99.7 $\pm$ 7.7	99.3 $\pm$ 3.2	96.4 $\pm$ 8.3
Alpha (1.0)	95.9 $\pm$ 7.5	101.2 $\pm$ 8.6	86.1 $\pm$ 6.6	102.3 $\pm$ 4.4	76.6 $\pm$ 7.1	88.4 $\pm$ 2.0
Alpha (3.0)	39.2 $\pm$ 12.3	56.7 $\pm$ 8.8	36.9 $\pm$ 11.9	59.3 $\pm$ 10.7	35.6 $\pm$ 11.3	57.6 $\pm$ 10.2
Delta (1.0)	89.4 $\pm$ 5.7	99.8 $\pm$ 5.7	90.1 $\pm$ 1.9	95.0 $\pm$ 3.0	91.0 $\pm$ 2.7	103.0 $\pm$ 2.5
Delta (5.0)	102.9 $\pm$ 4.1	94.8 $\pm$ 2.8	91.9 $\pm$ 1.7	92.0 $\pm$ 3.0	89.5 $\pm$ 3.1	88.1 $\pm$ 6.8
Delta (10.0)	993.4 $\pm$ 2.0	81.9 $\pm$ 2.0	90.6 $\pm$ 6.6	71.9 $\pm$ 5.0	96.0 $\pm$ 5.1	82.3 $\pm$ 5.2

^a SEA in 10 μl of PBS was added to 2 ml of BME plus 10% fetal calf serum in 35-mm wells of Henle 407 cell monolayers 24 h before evaluating amino acid transport. After the 24-h preincubation period, the cells were rinsed twice with HBSS. Toxin(s) at the concentrations listed was added in 2 ml of HBSS for 30 min and aspirated; then 2 ml of labeled amino acid solution containing 1 mM AIB (1 $\mu\text{Ci}/\text{ml}$) and 1 mM lysine (1 $\mu\text{Ci}/\text{ml}$) was added for 20 min of incubation at 37°C. Protein and radioactivity were determined as described in footnote a, Table 2. The values listed are the means of three to six determinations $\pm$ standard deviation. Statistical analysis indicated no significant interaction for combined enterotoxin and hemolytic toxin treatment ($P > 0.01$).

tivity of the hemolytic toxin. No synergism between the enterotoxin and the hemolytic toxins was evident.

On the basis of these data, we conclude that SEA caused no overt membrane damage of mammalian cells or modification of the membrane transport systems evaluated. The enterotoxin also did not predispose the plasma membrane of Henle 407 cells to subsequent damage by other staphylococcal toxins.

As another index for detecting cytotoxicity of SEA, macromolecular synthesis by the cell cultures exposed to the toxin was assessed. SEA had no effect on synthesis of protein, RNA, or DNA when incubated with Henle 407 cells for periods of between 2 and 24 h (Table 4). Thus, it may be concluded that SEA, even at concentrations exceeding physiological doses, does not alter macromolecular synthesis of the intestinal epithelial cells.

DISCUSSION

Our observations show clearly that two staphylococcal enterotoxin serotypes exert no measurable direct cytotoxic action on the mammalian cell cultures tested. Assays of membrane leakage and membrane functional integrity (i.e., intact amino acid transport systems) demonstrate that staphylococcal enterotoxin does not qualify as a microbial cytotoxin. Studies revealing normal macromolecular synthesis by mammalian cells after exposure to high concentrations of enterotoxin also do not support a mechanism involving direct toxicity to gastrointestinal tissues. It was also established that enterotoxin does not act synergistically to augment inhibitory effects of staphylococcal alpha- or delta-toxin on AIB or lysine uptake by Henle 407 cells. The evidence

for absence of synergy is compelling, since the assay used was shown to be sufficiently sensitive to detect synergism between alpha- and delta-toxins. The data presented in this report showing that staphylococcal enterotoxins do not behave as typical cytotoxins alone or in combination with known cytotoxins of staphylococcal origin are significant, for they mandate that other than direct mechanisms of toxicity be evaluated. In spite of considerable efforts, the biochemical basis of staphylococcal food poisoning remains an unresolved issue.

In addition to the recognized role of staphylococcal enterotoxin in food-associated gastroenteritis, enterotoxin has been detected in staphylococcal lesions. In a clinical study, approximately 50% of *S. aureus* isolates from patients with skin lesions were found to be enterotoxin producers (24). Josefczyk (7) reported that staphylococcal infections frequently result in detectable levels of serum antitoxin. This suggests that enterotoxin is disseminated systemically from staphylococcal lesions. Thus, consideration for a role of enterotoxin in staphylococcal infections becomes potentially significant. Indeed, enterotoxin administered intravenously to monkeys produces profound effects, including emesis, diarrhea, and, at higher doses, shock, fever, and death (1). Thus, staphylococcal enterotoxins may exert important effects during infection not appreciated because these entities are currently classified as enterotoxins.

The recent recognition that enterotoxins act as potent immunomodulating agents (13, 23) at extremely low concentrations (9) raises the possibility that they may act via mediation of immunological phenomena and not via a directly expressed cytotoxicity.

TABLE 4. Effect of SEA on synthetic capabilities of Henle 407 cells^a

Toxin or inhibitor	Macromolecular synthesis (% of control)					
	[³ H]leucine		[³ H]uridine		[³ H]thymidine	
	0 h	24 h	2 h	24 h	4 h	24 h
None (PBS)	100 ± 14	100 ± 17		100 ± 9	100 ± 15	100 ± 18
SEA						
0.1 µg/ml	100 ± 9	88 ± 9	99 ± 8	103 ± 4	100 ± 25	
1.0 µg/ml	84 ± 12	96 ± 9	95 ± 6	102 ± 3	89 ± 27	89 ± 41
10 µg/ml	94 ± 37	88 ± 7	98 ± 1	108 ± 6	73 ± 24	110 ± 48
<i>Pseudomonas</i> toxin						
10 µg/ml	30 ± 7 ^c					
50 µg/ml	11 ± 4 ^c					
Actinomycin D, 1.0 µg/ml				10 ± 1		
Mitomycin C, 30 µg/ml					26 ± 2 ^c	4 ± 1

^a Nearly confluent Henle 407 cells were treated with toxin for the times shown by aspirating spent medium and replacing with fresh BME containing 1 µCi of the indicated label per ml and toxin at the concentration listed. Incorporation into macromolecules was determined as described in Materials and Methods.

^c Significantly different from control (PBS).

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SPECIFIC RECEPTOR BINDING OF STAPHYLOCOCCAL ENTEROTOXIN IS
BY MURINE SPLENIC LYMPHOCYTES

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ABSTRACT

A reliable assay to measure specific binding of ^{125}I -staphylococcal enterotoxin A (^{125}I -SEA) by murine spleen cells is described. Toxin binding by lymphocytes was specific in that it was inhibited by unlabelled staphylococcal enterotoxin A (SEA) but not by unrelated proteins. The biological activity of SEA (T lymphocyte mitogenesis) correlated with toxin binding to splenic lymphocytes. In the presence of high concentrations of ^{125}I -SEA, specific binding increased rapidly and approached saturation after two hours. Toxin binding was sensitive to temperature and pH and was directly proportional to the concentration of spleen cells in the incubation mixture. A single class of toxin binding sites with an apparent equilibrium dissociation constant (K_d) of $8 \times 10^{-7}\text{M}$ and numbering 3600 sites per cell was estimated. SEA and the antigenically distinct SEB and SEE in excess competitively inhibited binding of ^{125}I -SEA to mouse spleen cells. The data suggest a common class of binding sites for the three staphylococcal enterotoxins.

INTRODUCTION

Staphylococcal enterotoxins comprise a group of five antigenically distinct proteins of MW 28,500 secreted during growth by a significant percentage of Staphylococcus aureus isolates (1). The toxins elicit a variety of biological activities including induction of diarrhea and emesis in primates (1). They also act as polyclonal mitogens (13) and depress antibody production (20). In a study comparing the mitogenicity induced by the polyclonal T-lymphocyte mitogens concanavalin A (Con A), phytohemagglutinin (PHA) and staphylococcal enterotoxin A (SEA), Langford, et al. (11) estimated that SEA is the most potent mitogen discovered to date. It has also been suggested that mitogenic substances of staphylococcal origin may induce lymphotoxin synthesis (22). Thus, one may speculate that one or more lymphokines may contribute in part to manifestations of gastrointestinal and systemic toxicity. The significant effects of staphylococcal enterotoxins on antibody (20), interferon (11) and migration inhibition factor production (9) as well as toxin suppression of allograft rejection (14) indicate that staphylococcal enterotoxins may be important as immunologic mediators in addition to any direct toxicity they may exert.

There is evidence that specific cell receptors play a critical role in the stimulation of mitogenesis induced by polyclonal lymphocyte mitogens (2,12,15). A previous study (23) provides indirect evidence for presence of specific staphylococcal enterotoxin receptors on lymphocyte membranes. The present study provides direct evidence for specific binding of SEA by spleen cells as the first step in lymphocyte mitogenesis. It also demonstrates that SEA associates with lymphocytes by specific ligand-receptor binding. Additionally, the binding characteristics of SEB and SEE by murine spleen cells are similar to those of SEA. The data suggest that this group of antigenically distinct but biologically similar microbial toxins mediate mitogenic stimulation via common receptors on

the lymphocyte surface.

MATERIALS AND METHODS

Murine spleen cells

Spleen cells were obtained from 6-12 week old C57B1/6 (C57) female mice purchased from Laboratory Supply Company, Indianapolis, Indiana. Spleens were aseptically removed and the spleen cells were teased from the organ capsule into a 35 mm tissue culture dish containing RPMI 1640 tissue culture medium (Microbiological Associates, Walkersville, MD.). Cells were washed and resuspended in RPMI 1640 + 10% fetal calf serum (FCS) before use.

Isotopes

Isotopes were purchased from New England Nuclear Boston, MA: (methyl- ^3H)-thymidine (6.7 Ci/mmol); carrier free Na ^{125}I (17 Ci/mg) in 0.1 N sodium hydroxide.

Staphylococcal enterotoxins

Staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB) and staphylococcal enterotoxin E (SEE) were purified by published methods (18,19).

Henle 407 cells

The Henle 407 embryonic human intestinal epithelial cell line (ATCC-CL6) was obtained from the American Type Culture Collection, Rockville, MD. This cell line was established originally by Dr. W. Henle from jejunum and ileum of a two month human embryo. Gartler (6) has suggested that this cell line is one of several which may now be contaminated with HeLa cells and thus the relationship of Henle 407 to human intestinal cells is uncertain. The cells were grown in Eagles' basal medium (Gibco, Grand Island, New York) and 10% FCS. The cells were detached from the surface by incubation with 0.1% EDTA in phosphate buffered saline, washed and resuspended in RPMI 1640 + 10% FCS.

Mitogenesis assay. To evaluate early events in the process leading to mitogenesis, it was necessary to develop an assay in which contact with toxin (mitogen) was of limited duration. Spleen cells (3×10^6) suspended in 0.5 ml RPMI 1640 + 10% FCS were added to 0.5 ml of medium containing 20 μ g/ml SEA in a 1.5 ml sterile polypropylene vial (Kew Scientific, Columbus, Ohio). The tubes were gassed with 10% CO₂ and incubated at 37°C for 3 hrs after which the vials were centrifuged at 1000x g for 5 min., washed twice and resuspended in RPMI 1640. Cells were counted using a Coulter Counter Model ZB (Coulter Electronics, Hialeah, FLA) and lymphocyte viability was evaluated by trypan blue dye exclusion. Cells were diluted in RPMI 1640 + 10% FCS and dispensed into 96-well microtiter plates (Costar Division of Bellco Glass Co., Vineland, N.J.) at a concentration of 10^5 cells/well. Incubation of microtiter plates at 37°C in 10% CO₂ in air without additional mitogen was continued for a total of 48 hrs, the last 4 hrs of which cells were pulsed with 0.5 μ Ci/well of ³H-thymidine.

Cells were collected on glass fiber filters by vacuum, washed with saline and finally with 95% ethanol. Filter pads were placed in scintillation vials with 10 ml Scintiverse scintillation fluid (Fisher Scientific, Cincinnati, OH) and counted. To measure temperature dependence of binding, the initial 3 hr incubation in the presence of SEA was carried out at 4°C, 22°C, or 37°C before the cells were washed free of nonassociated toxin and resuspended in RPMI 1640. Incubation was then continued for a total of 48 hrs. Experiments to determine the effect of pH on the interaction of spleen cells and SEA were performed in a modified incubation solution. SEA (2x concentrated) was dissolved in RPMI 1640 with 100 mM sodium phosphate buffer at pH 6.0, 6.5, 7.0, 7.5, or 8.0. An aliquot of spleen cells (6×10^6 /ml) in RPMI 1640 + 10% FCS was added to the toxin in modified RPMI 1640. Spleen cells were incubated at each pH for 3 hrs at 37°C in the presence of SEA. Cells were then washed and incubation was continued in

the absence of toxin. Incorporation of ^3H -thymidine during the last 4 hr of incubation was measured as described above.

Trypsin treatment of spleen cells. Washed spleen cells ($3 \times 10^6/\text{ml}$) were incubated for 30 min at 37°C in HBSS pH 7.4 containing 1.0 mg/ml trypsin (281 $\mu\text{g}/\text{mg}$; Worthington Biochemicals Corp., Freehold, N.J.). Cells were then used in the SEA mitogenesis or the toxin binding assays.

Iodination of SEA. SEA was labelled with ^{125}I as described by Kauffman and Johnson (10). Ten μg SEA was labelled by reaction with 10 mCi of carrier free Na^{125}I in 100 μl total volume containing 5 μg chloramine T and phosphate buffer. Labelled ^{125}I -SEA was recovered from a Sephadex G-25 column in phosphate buffered saline containing 0.1% bovine serum albumin. The ^{125}I -SEA was sterilized by filtration and stored in 250 μl aliquots at -70°C . Iodination of ^{125}I -SEA was achieved to a level of one iodine per molecule of protein (70-100 $\mu\text{Ci}/\mu\text{g}$). ^{125}I -SEA was used within 2 weeks of iodination although no loss of biological activity was detected for 2 months. Biological activity of ^{125}I -SEA determined in mitogen assays by direct comparison with uniodinated SEA revealed no differences in mitogenic potency.

Assay of ^{125}I -SEA binding to lymphoid cells. Binding of ^{125}I -SEA to spleen cells was carried out by incubating 0.5 ml of cells ($2 \times 10^7/\text{ml}$) at room temperature (23°C) for 3 hr in complete medium containing 0.125 $\mu\text{g}/\text{ml}$ ^{125}I -SEA or 0.125 $\mu\text{g}/\text{ml}$ ^{125}I -SEA plus 40 $\mu\text{g}/\text{ml}$ SEA (control for non-specific binding) and dispensed in 1.5 ml plastic vials. Cells were shaken periodically and at the conclusion of the incubation period, four-100 μl samples were removed and layered over 200 μl of silicon oil (Versilube F-50, Harwich Inc., Chicago, IL.) in 400 μl polyethylene vials (Kew Scientific, Columbus, OH). The vials were centrifuged in a Beckman Microfuge for 30 sec at $12,000 \times g$ and rapidly frozen in dry ice-ethanol. The bottom of each vial containing the cell pellet was cut

off and counted in a gamma counter.

RESULTS

The mitogenic activity of SEA was characterized by washing spleen cells free of unbound toxin after 3 hr and incubating at 37°C in the absence of additional SEA for a total of 48 hr to allow full expression of mitogenic potential. The data in Table 1 show that mitogenic stimulation was identical for cells which were exposed to SEA for only the first 3 hr or exposed to toxin continuously for 48 hr. This assay was also used to determine the effect of temperature and pH on toxin binding and subsequent mitogenic stimulation. The SEA mitogenesis assay was employed in conjunction with a toxin binding assay to establish the correlation between the extent of toxin binding and mitogenic stimulation.

An assay was developed to detect the specific binding of iodinated SEA to spleen cells. The kinetics of ^{125}I -SEA association with mouse spleen cells at 23°C are shown in Fig. 1. Specific toxin binding reached saturation within 2 hr. Non-specific association of toxin is defined as the amount of cell associated ^{125}I -SEA observed in the presence of 40 µg/ml unlabelled SEA. The amount of non-specifically associated ^{125}I -SEA increased linearly with time suggesting pinocytic uptake of toxin. The binding of ^{125}I -SEA was a linear function of spleen cell concentration (Fig.2). The effect of the iodinated toxin concentration on the total amount of specific binding is shown in Fig.3. Specific binding increased as the concentration of ^{125}I -SEA was increased and approached saturation at 1.5 µg/ml ^{125}I -SEA. These data were replotted according to the method of Scatchard (16) as shown in Fig. 4. The slope of the Scatchard plot indicates a single class of binding sites with an apparent dissociation constant (K_d) of 8×10^{-7} M. The X-intercept of the plot corresponds to an estimated value of 3600 binding sites per spleen cell. Since splenocytes consist of a mixture of T-lymphocytes, B-lymphocytes, and lesser

numbers of macrophages the value calculated for the number of binding sites per cell represents an average for a mixed cell population. The binding sites per cell for specific cell types will require similar studies carried out with purified T-and B-cells. Experiments not shown here indicate that macrophages do not possess specific receptor sites for SEA but rather take up toxin via non adsorbtive pinocytosis (manuscript in preparation).

The specific binding of ^{125}I -SEA was inhibited not only by unlabelled SEA but also by the antigenically dissimilar staphylococcal enterotoxins SEB and SEE (Fig.5). At low concentrations of unlabelled toxin the amount of ^{125}I -SEA specifically bound was greater (statistically significant at $p < .05$) than specific binding in the absence of unlabelled toxin, suggestive of positive cooperativity. SEA and SEE were equally inhibitory at intermediate concentrations (1.0-10 $\mu\text{g/ml}$) while SEB was a less effective inhibitor of ^{125}I -SEA binding at intermediate and high concentrations. The extent of inhibition of ^{125}I -SEA binding by homologous or heterologous unlabelled toxin correlated with mitogenic potency. SEA and SEE inhibited binding of ^{125}I -SEA and stimulated mitogenesis to the same extent. In contrast, SEB was less inhibitory for SEA binding and also was a weaker mitogen (Table 3). These data indicate that these antigenically distinct staphylococcal enterotoxins recognize similar or identical binding sites on mouse spleen cells.

The binding of ^{125}I -SEA and the stimulation of mitogenesis were correspondingly affected by the pH of the incubation medium (Fig. 6). Specific toxin binding and mitogenic stimulation were maximal at pH 6.5 and declined at higher or lower pH's. The correlation between binding and mitogenic stimulation of lymphocytes indicates that the toxin binding leads to a biological response. Specific binding increased linearly with temperature (Fig. 7), but mitogenic stimulation decreased slightly at higher temperatures. The quantity of toxin

binding and mitogenic stimulation observed after trypsin treatment of the spleen cell is shown in Table 3. Trypsinization of spleen cells reduced mitogenic stimulation by SEA but did not inhibit binding of ^{125}I -SEA. Specific binding of ^{125}I -SEA to a human embryonic intestinal epithelial cell line (Henle 407) could not be demonstrated (Table 4). Although a significant amount of ^{125}I -SEA associated with the Henle 407 cells, excess unlabelled SEA did not compete with iodinated SEA associating with the cells. This would suggest an absence of SEA receptors on the surface of the Henle 407 cells.

DISCUSSION

A relatively simple, reliable assay to detect spleen cell binding of staphylococcal enterotoxins has been described. Saturation of binding between 2 and 3 hr after initiation of ^{125}I -SEA-lymphocyte interaction corresponded to the time required for induction of maximum mitogenic stimulation (Table 1). Saturation of specific binding at high ^{125}I -SEA concentration (Fig. 3) was demonstrable and relatively high affinity of the toxin for spleen cells is indicated by Scatchard analysis of the data (Fig. 4). Toxin binding increased linearly over a wide range of cell concentrations (Fig. 2). Three antigenically distinct staphylococcal enterotoxins; i.e., SEA, SEB, and SEE inhibited specific binding of ^{125}I -SEA in a dose dependent manner, but a high concentration of unrelated proteins (10% FCS) did not block toxin binding. In summary, these results demonstrate the specificity of ^{125}I -SEA binding by lymphocytes and confirm that ligand-receptor interaction correlates with a biological response of cells to toxin.

^{125}I -SEA binding to murine spleen cells did not correlate precisely with mitogenic stimulation by toxin over a temperature range from 0°C to 37°C (Fig. 7). Toxin binding is more efficient at 37°C but greater mitogenic stimulation results when toxin binding is carried out at 4°C . A possible explanation is

obtained by an analysis of the data in Table 1 and Fig. 7. Table 1 shows that 1.0 $\mu\text{g/ml}$ SEA induces a greater mitogenic response than does 10 $\mu\text{g/ml}$ SEA. This high dose depression of mitogenesis was noted in several separate experiments. The mitogenic stimulation by SEA shown in Fig. 7 was achieved with 10 $\mu\text{g/ml}$ SEA. The decreased mitogenic response at this concentration as compared with the response when binding occurs at 4°C may result from more efficient toxin binding at 23°C and 37°C . The effective toxin concentration at higher temperatures thus, may lie in the range of high dose suppression whereas less efficient toxin binding at 4°C results in lesser but optimum amounts of SEA bound to achieve peak mitogenic stimulation. The data presented fulfill criteria for specific toxin binding to cell surface receptors. These criteria include chemical specificity, saturability, high affinity, and correlation with a measurable biological response by the cells (4).

The increase in specific binding of ^{125}I -SEA observed in the presence of low concentrations of unlabelled staphylococcal enterotoxins (Fig. 5) suggest positive cooperativity for SEA binding as has been observed for binding of plant lectins to lymphocytes. Prujansky, et al. (15) suggest that positive cooperativity of binding to lymphocytes occurs with mitogenic lectins but not with lectins which bind to lymphocytes but do not initiate a mitogenic response. The data shown in Fig. 7 suggest cooperative toxin binding, Scatchard plot analysis of data generated over a wider range of ^{125}I -SEA concentrations, however, is required to substantiate this observation. This is an important consideration since Prujansky, et al. (15) hypothesize that cooperative binding of mitogen reflects alterations in cell membrane architecture and may constitute an essential event in lymphocyte blastogenesis.

The data show that cell binding of SEA is not reduced by trypsin treatment but mitogenic stimulation is substantially reduced. Thus, the SEA binding site

appears to be trypsin resistant. Trypsinization may reduce mitogenic stimulation either by disruption of T and B cell interactions required for optimum mitogenic responses, or alternatively, by inactivation of membrane-associated proteins required for initiation of cell division. The absence of specific binding of ^{125}I -SEA to the Henle 407 cells is significant in that it may explain lack of cytotoxicity exhibited by enterotoxin for this cell line (3). Direct cytotoxicity has been difficult to substantiate in spite of claims that staphylococcal enterotoxins damage cells of the gastrointestinal epithelium (1,17). However, the high reactivity of enterotoxins in immunological phenomena mandates a reevaluation of how the toxins act in vivo. The immunological responses of human lymphocytes to staphylococcal enterotoxins are quite similar to those of mouse spleen cells (13), and thus it is likely that enterotoxin mitogenic stimulation of human lymphoid cells is also mediated by specific toxin receptors.

Staphylococcal enterotoxin stimulation of gut associated lymphoid tissue in the human gastrointestinal tract may play a significant role in the genesis of symptoms associated with food poisoning. Recently, intraepithelial mast cell-like T-lymphocytes containing histamine have been detected in intestinal tissue of mice (7) and humans (5). Since other lymphocyte mitogens are known to trigger histamine release from mast cells (8,21), one may speculate that SEA acts similarly. Release of this potent pharmacological agent within the gastrointestinal tract conceivably may relate to symptoms of staphylococcal food poisoning. Toxicity of staphylococcal enterotoxins may be expressed as an indirect effect mediated by toxic lymphokines and pharmacologically active amines released after lymphocyte stimulation; this is a possibility currently under investigation (D.L. Archer, personal communication).

In conclusion, the data presented here provide direct evidence for the presence of staphylococcal enterotoxin receptors on murine spleen cells. In

contrast, it was not possible to detect specific toxin binding by cells more closely related to intestinal epithelial cells which are often cited as potential target cells for direct cytopathic effects of staphylococcal enterotoxins (1,17).

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Table 1. Mitogenic response of murine splenic lymphocytes after 3 hr or 48 hr interaction with SEA

Time (hr)	Toxin Conc. ($\mu\text{g/ml}$)	^3H -thymidine Incorporated ($\bar{X} \pm \text{std. error}$)
3	0.0	1704 $\pm$ 129
3	0.1	11229 $\pm$ 516
3	1.0	17697 $\pm$ 3273
3	10.0	15743 $\pm$ 267
48	0.0	884 $\pm$ 99
48	0.1	17089 $\pm$ 1926
48	1.0	16384 $\pm$ 1083
48	10.0	15430 $\pm$ 546

Murine splenic lymphocytes were incubated with SEA at the concentration shown for 3 or 48 hr. Each value is the mean of 8 samples.

Table 2. Mitogenic stimulation of splenic lymphocytes by SEA, SEB or SEE

Toxin Conc. (μ g/ml)	CPM 3 H-Thymidine Incorporated		
	SEA	SEB	SEE
0.0	2962 $\pm$ 426		
0.0001	5991 $\pm$ 454	2992 $\pm$ 529	5602 $\pm$ 402
0.01	12375 $\pm$ 455	5713 $\pm$ 727	9006 $\pm$ 545
1.0	14261 $\pm$ 522	10149 $\pm$ 301	14003 $\pm$ 347

Splenic lymphocytes were incubated with SEA, SEB or SEE at the concentration indicated for 48 hr. Each value is the mean $\pm$ standard error for 4 replicate samples.

Table 3. Effect of trypsinization of spleen cells on the mitogenic response and specific binding of SEA

Trypsin Concentration*	CPM ^3H -thymidine incorporated $\bar{X} \pm \text{std. error}$	CPM of ^{125}I -SEA bound $\bar{X} \pm \text{std. error}$
none	6173 $\pm$ 300	1192 $\pm$ 139
1.0 mg/ml	1593 $\pm$ 116	1039 $\pm$ 137

*Spleen cells were treated for 30 min at 37°C with 1 mg/ml of trypsin. No significant differences in toxin binding values are apparent ($p > 0.01$). Values are those of 3 separate experiments.

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