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Specific Receptor Binding of Staphylococcal Enterotoxins by Murine Splenic Lymphocytes

STEPHEN BUXSER,[1]† PETER F. BONVENTRE,[1]* AND DOUGLAS L. ARCHER[2]

Department of Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267[1] and Division of Microbiology, Food and Drug Administration, Department of Health and Human Services, Cincinnati, Ohio 45226[2]

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We describe a reliable assay to measure the specific binding of ^{125}I-labeled staphylococcal enterotoxin A (SEA) by murine spleen cells. Toxin binding by lymphocytes was specific in that it was inhibited by unlabeled 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 2 h. Toxin binding was sensitive to temperature and pH and was directly proportional to the concentration of spleen cells in the incubation mixture. We estimated that there was a single class of toxin-binding sites, which had an apparent equilibrium dissociation constant (K_d) of 8×10^{-7} M and numbered 3,600 sites per cell. SEA and the antigenically distinct compounds staphylococcal enterotoxins B and E in excess competitively inhibited binding of [^{125}I]SEA to mouse spleen cells. Our data suggest a common class of binding sites for the three staphylococcal enterotoxins.

The staphylococcal enterotoxins are a group of five antigenically distinct proteins which are secreted during growth by a significant percentage of *Staphylococcus aureus* isolates (1). These 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 mitogenic activities induced by the polyclonal T-lymphocyte mitogens concanavalin A, phytohemagglutinin, 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 toxicities. 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 immunological mediators, in addition to any direct toxicity that they 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) provided indirect evidence for the presence of specific staphylococcal enterotoxin receptors on lymphocyte membranes, and the present study provides direct evidence for specific binding of SEA by spleen cells as the first step in lymphocyte mitogenesis. This study also demonstrates that SEA associates with lymphocytes by specific ligand-receptor binding. In addition, the binding characteristics of staphylococcal enterotoxin B (SEB) and staphylococcal enterotoxin E (SEE) by murine spleen cells are similar to the binding characteristics of SEA. Our data suggest that the members of 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- to 12-week-old C57BL/6 female mice purchased from Laboratory Supply Co., Indianapolis, Ind. Spleens were removed aseptically, and the spleen cells were teased from each organ capsule into a 35-mm tissue culture dish containing RPMI 1640 tissue culture medium (Microbiological Associates, Walkersville, Md.). The cells were washed and suspended in RPMI 1640 containing 10% fetal calf serum (FCS) before use.

† Present address: Department of Biochemistry, University of Massachusetts Medical School, Worcester, MA 01605.

Isotopes. The following isotopes were purchased from New England Nuclear Corp., Boston, Mass.: [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) and carrier-free Na^{125}I (17 Ci/mg) in 0.1 N sodium hydroxide.

Staphylococcal enterotoxins. SEA, SEB, and SEE were purified by previously described 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 W. Henle from the jejunum and ileum of a 2-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 cells to human intestinal cells is uncertain. These cells were grown in Eagle basal medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% FCS. The cells were detached from the growth surface by incubation with 0.1% ethylenediaminetetraacetate in phosphate-buffered saline, washed, and suspended in RPMI 1640 medium containing 10% FCS.

Mitogenesis assay. To evaluate the 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. A total of 3×10^6 spleen cells suspended in 0.5 ml of RPMI 1640 medium containing 10% FCS were added to 0.5 ml of medium containing 20 μg of SEA per ml in a 1.5-ml sterile polypropylene vial (Kew Scientific, Columbus, Ohio). Each tube was gassed with 10% CO_2, incubated at 37°C for 3 h, and then centrifuged at $1,000 \times g$ for 5 min, and the cells were washed twice and suspended in RPMI 1640 medium. The cells were counted by using a model ZB Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.), and lymphocyte viability was evaluated by trypan blue dye exclusion. Cells were diluted in RPMI 1640 medium containing 10% FCS and dispensed into 96-well microtiter plates (Costar Div., Bellco Glass, Inc., Vineland, N.J.) at a concentration of 10^5 cells per well. Then the microtiter plates were incubated at 37°C in 10% CO_2 in air without additional mitogen for a total of 48 h, during the last 4 h of which the cells were pulsed with 0.5 μCi of [^{3}H]thymidine per well.

Cells were collected on glass fiber filters by vacuum and then washed with saline and finally with 95% ethanol. Filter pads were placed in scintillation vials with 10 ml of Scintiverse scintillation fluid (Fisher Scientific Co., Cincinnati, Ohio) and counted. To measure the temperature dependence of binding, the initial 3-h incubation in the presence of SEA was carried out at 4, 22, or 37°C before the cells were washed free of nonassociated toxin and resuspended in RPMI 1640 medium. Incubation was then continued for a total of 48 h. Experiments to determine the effect of pH on the interaction between spleen cells and SEA were performed in a modified incubation solution. SEA (twice concentrated) was dissolved in RPMI 1640 medium containing 100 mM sodium phosphate buffer at pH 6.0, 6.5, 7.0, 7.5, or 8.0. A sample of spleen cells (6×10^6 cells per ml) in RPMI 1640 medium containing 10% FCS was added to the toxin in modified RPMI 1640 medium. Spleen cells were incubated at each pH for 3 h at 37°C in the presence of SEA. Cells were then washed, and incubation was continued in the absence of toxin. Incorporation of [^{3}H]thymidine during the last 4 h of incubation was measured as described above.

Trypsin treatment of spleen cells. Washed spleen cells (3×10^6 cells per ml) were incubated for 30 min at 37°C in Hanks balanced salt solution (pH 7.4) containing 1.0 mg of trypsin per ml (281 μg/mg; Worthington Diagnostics, Freehold, N.J.). Cells were then used in the SEA mitogenesis or the toxin-binding assay.

Iodination of SEA. SEA was labeled with ^{125}I as described by Kauffman and Johnson (10); 10 μg of SEA was reacted with 10 mCi of carrier-free Na^{125}I in 100 μl (total volume) containing 5 μg of chloramine-T and phosphate buffer. ^{125}I-labeled 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 portions at −70°C. Iodination of [^{125}I]SEA was achieved to a level of one iodine per molecule of protein (70 to 100 μCi/μg). [^{125}I]SEA was used within 2 weeks of iodination, although no loss of biological activity was detected for 2 months. The biological activity of [^{125}I]SEA as 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 performed by incubating 0.5 ml of cells (2×10^7 cells per ml) at room temperature (23°C) for 3 h in complete medium containing 0.125 μg of [^{125}I]SEA per ml or 0.125 μg of [^{125}I]SEA per ml plus 40 μg of SEA per ml (control for nonspecific binding) and dispensing into 1.5-ml plastic vials. The 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, Ill.) in 400-μl polyethylene vials (Kew Scientific). These vials were centrifuged in a Beckman Microfuge for 30 s 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 h and incubating them at 37°C in the absence of additional SEA for a total for 48 h to allow full expression of mitogenic potential. Table 1 shows that mitogenic stimulation activities were identical for cells which were exposed to SEA for only the first 3 h and cells which were exposed to toxin continuously for 48 h. This assay was also used to determine the effects of temperature and pH on toxin binding and subsequent mitogenic stimulation. The SEA mitogenesis assay was used in conjunction with a toxin-binding assay to establish the correlation between the extent of toxin binding and mitogenic stimulation.

We developed an assay to detect specific bind-

ing of iodinated SEA to spleen cells. Figure 1 shows the kinetics of [^{125}I]SEA association with mouse spleen cells at 23°C. Specific toxin binding reached saturation within 2 h. Nonspecific association of toxin was defined as the amount of cell-associated [^{125}I]SEA observed in the presence of 40 μg of unlabeled SEA per ml. The amount of nonspecifically 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 concentration of iodinated toxin 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 of [^{125}I]SEA per ml. These data were replotted according to the method of Scatchard (16), as shown in Fig. 4. The slope of the Scatchard plot indicated that there was a single class of binding sites, with an apparent dissociation constant (K_d) of 8×10^{-7} M. The X-intercept of the plot corresponded to an estimated value of 3,600 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 represented an average for a mixed cell population. To determine the number of binding sites per cell for specific cell types, similar studies carried out with purified T- and B-cells will be necessary. We also found that macrophages do not possess specific receptor sites for SEA but rather take up toxin via nonadsorptive pinocytosis (manuscript in preparation).

The specific binding of [^{125}I]SEA was in-

TABLE 1. *Mitogenic responses of murine splenic lymphocytes after 3- and 48-h interactions with SEA*[a]

Time (h)	Toxin concn (μg/ml)	Amt of [^{3}H]thymidine incorporated (cpm)
3	0.0	1,704 ± 129
3	0.1	11,229 ± 516
3	1.0	17,697 ± 3,273
3	10.0	15,743 ± 267
48	0.0	884 ± 99
48	0.1	17,089 ± 1,926
48	1.0	16,384 ± 1,083
48	10.0	15,430 ± 546

[a] Murine splenic lymphocytes were incubated with SEA at the concentrations shown for 3 or 48 h. Each value is the mean ± standard error of eight samples.

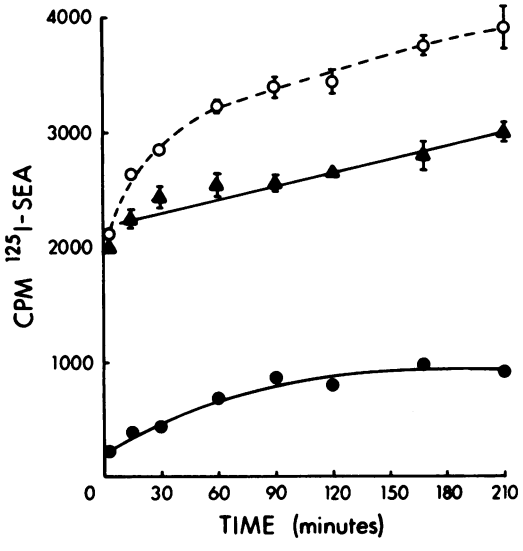

FIG. 1. *Time course of binding of [^{125}I]SEA to C57BL/6 mouse spleen cells. [^{125}I]SEA at a concentration of 0.125 μg/ml was incubated with 2×10^7 spleen cells per ml at 23°C in RPMI 1640 medium containing 10% FCS. Cell-associated [^{125}I]SEA was separated from supernatants by centrifuging 100-μl samples through 200 μl of silicon oil. The difference between total binding (○) and nonspecific binding (▲) is shown as specific binding (●). Nonspecific binding was determined by incubating cells with [^{125}I]SEA as described above in the presence of 40 μg of unlabeled SEA per ml. The values shown are means ± standard deviations of four replicate samples. The results shown are from three separate experiments.*

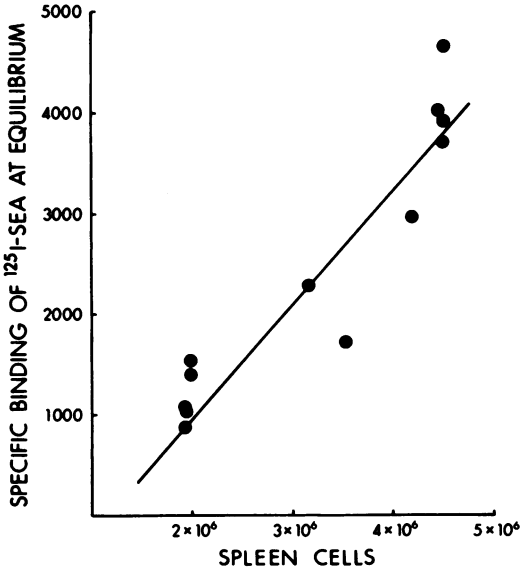

FIG. 2. *Effect of cell concentration on SEA binding by C57BL/6 mouse spleen cells. Spleen cells were incubated for 3 h at 23°C with [^{125}I]SEA as described in the legend to Fig. 1. Each value shown is the mean of four determinations from an independent experiment. The standard error for each value was within 10% of the mean. The line shown was obtained by linear regression ($r = 0.93$).*

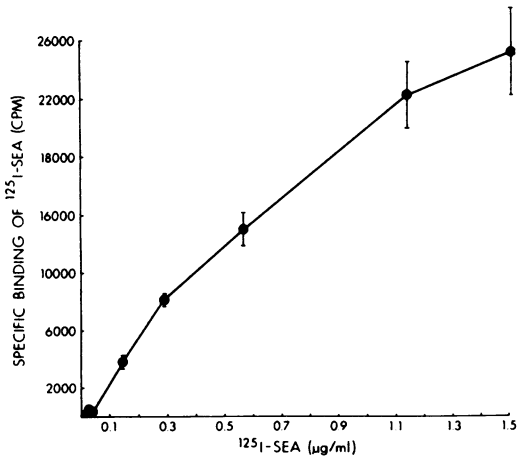

FIG. 3. *Effect of [^{125}I]SEA concentration on specific binding to C57BL/6 mouse spleen cells. Spleen cells (3 × 10^7 cells per ml) were incubated with [^{125}I]SEA for 3 h at 23°C in RPMI 1640 medium containing 10% FCS. The values are means of four samples and are corrected for nonspecific binding.*

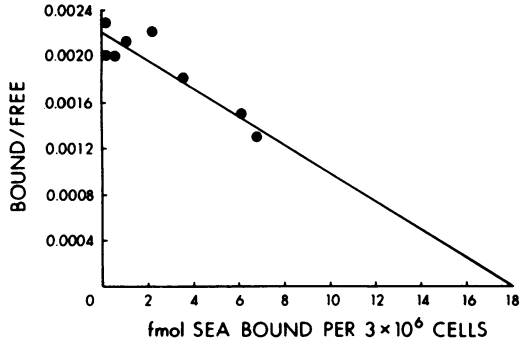

FIG. 4. *Scatchard plot of SEA binding to mouse spleen cells. The data from Fig. 3 were replotted by the method of Scatchard (16). The line was fitted by linear regression (r = 0.90). The x-intercept corresponds to an estimated value of 3,600 receptor sites per cell, and the slope indicates an apparent K_d of 8 × 10^{-7} M.*

hibited not only by unlabeled SEA but also by the antigenically dissimilar toxins SEB and SEE (Fig. 5). At low concentrations of unlabeled toxin, the amount of [^{125}I]SEA specifically bound was greater (statistically significant at $P < 0.05$) than the specific binding in the absence of unlabeled toxin, suggesting positive cooperativity. SEA and SEE were equally inhibitory at intermediate concentrations (1.0 to 10 μg/ml), whereas 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 unlabeled 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 2). 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 mitogensis 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 indi-

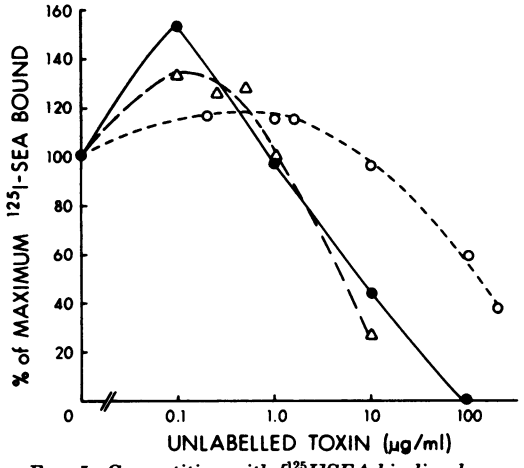

FIG. 5. *Competition with [^{125}I]SEA binding by unlabeled SEA, SEB, and SEE. C57BL/6 mouse spleen cells (3.5 × 10^7 cells per ml) were incubated in RPMI 1640 medium containing 10% FCS, 0.125 μg of [^{125}I]-SEA per ml, and unlabeled SEA (●), SEB (○), or SEE (△) at different concentrations. Nonspecific binding was the difference between the amount of [^{125}I]SEA bound in the absence of unlabeled competitor and the amount of [^{125}I]SEA bound in the presence of 100 μg of SEA per ml. The values shown are the means from three experiments (four replicates per experiment). The standard errors were all within 20% of the mean values.*

TABLE 2. *Mitogenic stimulation of splenic lymphocytes by SEA, SEB, and SEE*[a]

Toxin concn (μg/ml)	Amt of [^{3}H]thymidine incorporated (cpm) with:		
	SEA	SEB	SEE
0.0	2,962 ± 426		
0.0001	5,991 ± 454	2,992 ± 529	5,602 ± 402
0.01	12,375 ± 455	5,713 ± 727	9,006 ± 545
1.0	14,261 ± 522	10,149 ± 301	14,003 ± 347

[a] Splenic lymphocytes were incubated with SEA, SEB, or SEE at the concentrations indicated for 48 h. Each value is the mean ± standard error for four replicate samples.

cated that toxin binding leads to a biological response. Specific binding increased linearly with temperature (Fig. 7), but mitogenic stimulation decreased slightly at higher temperatures. Table 3 shows quantities of toxin binding and mitogenic stimulation observed after trypsin treatment of spleen cells. 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 unlabeled SEA did not compete with the iodinated SEA that associated with the cells. This suggests an absence of SEA receptors on the surfaces of Henle 407 cells.

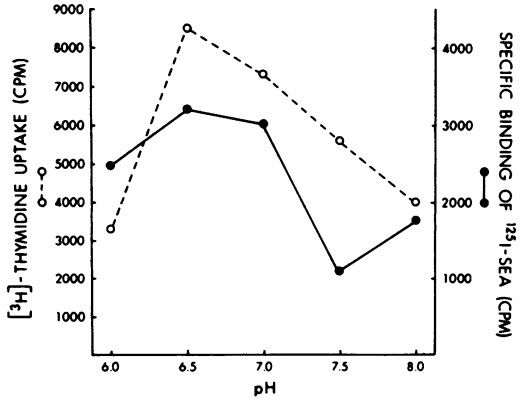

FIG. 6. *Effect of pH on the binding of [^{125}I]SEA (●) and on mitogenic stimulation (○). Specific binding was determined by incubating 4.5 × 10^7 cells with 0.125 μg of [^{125}I]SEA per ml and of 40 μg of SEA per ml at different pH's. After 3 h of incubation at 23°C, four 100-μl samples were centrifuged through silicon oil. Mitogenic stimulation was determined for spleen cells incubated with 10 μg of SEA per ml. The values shown are the means of values from two experiments, with 8 replicates at each pH for each experiment. The standard errors were all within 10% of the mean values.*

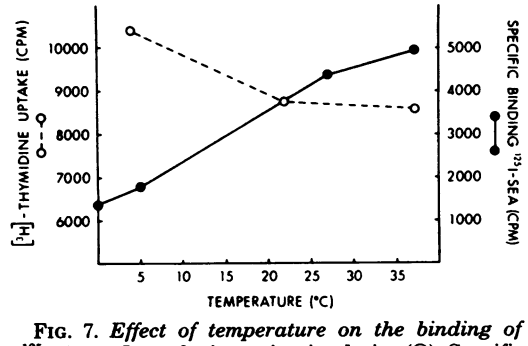

FIG. 7. *Effect of temperature on the binding of [^{125}I]SEA (●) and mitogenic stimulation (○). Specific binding was determined with samples of 4.5 × 10^7 cells per ml incubated at different temperatures for 3 h. The values shown are means of four samples. The standard errors were all within 10% of the means. The mitogenic response values shown are means from two separate experiments, with four samples processed at each temperature.*

DISCUSSION

We describe a relatively simple, reliable assay to detect spleen cell binding of staphylococcal enterotoxins. Saturation of binding between 2 and 3 h 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 concentrations (Fig. 3) was demonstrable, and relatively high affinity of the toxin for spleen cells was indicated by a 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 (SEA, SEB, and SEE) inhibited specific binding of [^{125}I]SEA in a dose-dependent manner, but a high concentra-

TABLE 3. *Effect of trypsinization of spleen cells on the mitogenic response and specific binding of SEA*[a]

Trypsin concn (mg/ml)	Amt of [^{3}H]thymidine incorporated (cpm)	Amt of [^{125}I]SEA bound (cpm)
None	6,173 ± 300[b]	1,192 ± 139[b]
1.0	1,593 ± 116	1,039 ± 137

[a] Spleen cells were treated for 30 min at 37°C with 1 mg of trypsin per ml. No significant differences in toxin-binding values were apparent ($P > 0.01$). Values are from three separate experiments.

[b] Mean ± standard error.

TABLE 4. *Absence of specific binding of [^{125}I]SEA to Henle 407 human intestinal epithelial cells*[a]

Time (min)	360-fold excess of unlabeled SEA	Amt of [^{125}I]SEA (cpm)
15	−	5,124 ± 273
15	+	5,369 ± 106
60	−	5,279 ± 138
60	+	5,195 ± 272
120	−	5,493 ± 189
120	+	5,209 ± 158

[a] Henle 407 cells grown in monolayers were suspended at a concentration of 5 × 10^6 cells per ml in RPMI 1640 medium containing FCS and incubated at 23°C with iodinated SEA alone or with iodinated SEA (0.125 μg/ml) plus a 320-fold excess of unlabeled SEA. Cells were then centrifuged through silicon oil. Values are means ± standard deviations of four samples.

tion 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 the 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 to 37°C (Fig. 7). Toxin binding was more efficient at 37°C, but greater mitogenic stimulation resulted when toxin binding was performed at 4°C. A possible explanation for this was obtained by an analysis of the data in Table 1 and Fig. 7. Table 1 shows that 1.0 μg of SEA per ml induced a greater mitogenic response than 10 μg of SEA per ml. This high-dose depression of mitogenesis was observed in several separate experiments. The mitogenic stimulation by SEA shown in Fig. 7 was achieved with 10 μg of SEA per ml. The decreased mitogenic response at this concentration compared with the response when binding occurred at 4°C may have resulted from more efficient toxin binding at 23 and 37°C. Thus, the effective toxin concentration at higher temperatures may lie in the range of high-dose suppression, whereas less efficient toxin binding at 4°C results in lower but optimum amounts of SEA bound to achieve peak mitogenic stimulation. The data presented above fulfill the 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 cells (4).

The increases in the specific binding of [^{125}I]-SEA observed in the presence of low concentrations of unlabeled 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 in Fig. 7 suggest cooperative toxin binding; however, Scatchard plot analysis of data generated over a wider range of [^{125}I]SEA concentrations will be required to substantiate this observation. This is an important consideration since Prujansky et al. (15) hypothesized that cooperative binding of mitogen reflects alterations in cell membrane architecture and may constitute an essential event in lymphocyte blastogenesis.

Our data show that cell binding of SEA is not reduced by trypsin treatment but that mitogenic stimulation is reduced substantially. Thus, the SEA-binding site appears to be trypsin resistant. Trypsinization may reduce mitogenic stimulation either by disruption of the T- and B-cell interactions required for optimum mitogenic responses or, alternatively, by inactivation of the membrane-associated proteins required for initiation of cell division. The absence of specific binding of [^{125}I]SEA to Henle 407 cells is significant in that this may explain the lack of cytotoxicity exhibited by enterotoxin for this cell line (3). Direct cytotoxicity has been difficult to substantiate despite 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 tissues 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 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 possibility is currently being investigated (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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