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Biological Effects of Staphylococcal Enterotoxin A on Human Peripheral Lymphocytes

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The mitogenicity, ability to induce immune interferon, and relationship between interferon synthesis and cell proliferative response were studied using human peripheral lymphocytes stimulated by staphylococcal enterotoxin A (SEA), phytohemagglutinin-P (PHA-P), and concanavalin A (ConA). Maximum cell proliferative responses ([³H]thymidine incorporation) and protein synthesis (¹⁴C-amino acid incorporation) occurred on days 3 and 4, respectively, after stimulation by each of the three mitogens. Maximal immune interferon levels were found 3 or 4 days after mitogen stimulation. SEA-treated cultures produced approximately three times more interferon than did cultures stimulated with PHA-P or ConA. Furthermore, SEA stimulated maximal cell proliferation over a much broader concentration range than did PHA-P and ConA (SEA, 10^{-5} to 10^2 µg/ml; PHA-P, 10^1 to 10^2 µg/ml; ConA, 10^1 to $10^{1.5}$ µg/ml). Interferon was also produced at maximal or near maximal levels over a broad concentration range of SEA (10^{-2} to 10^2 µg/ml). Also, we found that inhibition of mitogen-induced DNA and protein synthesis to control levels by mitomycin C or cytosine arabinoside partially reduced interferon production. The DNA inhibitor studies indicate that immune interferon synthesis occurs maximally in association with at least some proliferative response and that submaximal levels of interferon production occur in mitogen-treated cultures in the absence of detectable proliferation. The ability of SEA to stimulate maximal DNA and immune interferon synthesis at concentrations of 3.5×10^{-13} M and 3.5×10^{-10} M, respectively, puts it in a potency range similar to that of hormones. Thus, SEA may play an important role in gut immunity and *Staphylococcus aureus* infections at concentrations well below those required for emetic effects.

The staphylococcal enterotoxins are the causative agents of staphylococcal food poisoning (1). Recently, we have shown that staphylococcal enterotoxin A (SEA) is a T-lymphocyte mitogen, induces interferon (immune interferon), and inhibits the in vitro antibody response via suppressor T-cell activation in the mouse system (10, 11, 16). SEA is more potent than concanavalin A (ConA) or phytohemagglutinin-P (PHA-P) in inducing interferon and suppressing the in vitro antibody response in mouse spleen cell cultures. Furthermore, SEA was found to exhibit maximal mitogenic activity and interferon production over a broad concentration range (10^{-2} to 10^1 µg/ml) in contrast to much narrower concentration ranges for ConA and PHA-P (11). The relative mitogenic activity and interferon inducibility of SEA for human peripheral lymphocytes has not been determined. Thus, one objective of this study was to determine the relative mitogenicity and interferon inducibility of SEA, ConA, and PHA-P in the human peripheral lymphocyte system, because such data

may shed light on the possible role of SEA in gut immunity and *Staphylococcus aureus* infections.

It has been reported that mitogen induction of immune interferon (a lymphokine) is independent of the proliferative response (3, 22). Furthermore, development of both helper and suppressor cell activities in the in vitro antibody response of mouse spleen cells has been reported to be dissociable from DNA synthesis (19, 20). The second objective of this study, therefore, was to determine the relationship of SEA-induced DNA and protein synthesis to SEA-induced immune interferon synthesis in human peripheral lymphocytes. This information may lead to a better understanding of the mechanism of SEA-induced suppression of the immune response.

MATERIALS AND METHODS

Cell preparation. Blood (30 to 40 ml) was collected in acid-citrate-dextrose solution by venipuncture from 20 healthy volunteers. Peripheral blood lymphocytes

were isolated by the Ficoll-Hypaque gradient method (2, 15). All preparations contained more than 90% monocytic leukocytes, of which more than 95% were viable by trypan blue exclusion. The Ficoll-Hypaque gradient-separated lymphocytes were suspended and cultured in RPMI medium (10^6 lymphocytes per ml) containing L-glutamine (Microbiological Associates, Walkersville, Md.) and supplemented with 10% fetal bovine serum and antibiotics (100 U of penicillin per ml, 100 μg of streptomycin per ml, and 100 μg of gentamicin per ml). After the treatments indicated below, all cultures were incubated at 37°C in rocking (6 to 8 rocks per min) chambers containing a defined atmosphere of 10% CO_2, 7% O_2, and 83% N_2. Cultures were fed by the daily addition of nutritional mixture (0.05 ml) and fetal bovine serum (0.025 ml) (13).

Mitogens and lymphocyte stimulation. ConA, twice crystallized, was obtained from ICN Pharmaceuticals, Cleveland, Ohio. It was stored at room temperature. SEA was produced and purified by the Microbial Biochemistry Branch, Division of Microbiology, Food and Drug Administration, Cincinnati, Ohio (1). SEA migrated as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis at a molecular weight of approximately 28,000 and produced a single, symmetrical peak on Sephadex G-75. Purified PHA-P was obtained from Burroughs Wellcome, Research Triangle Park, N.C. Substocks of all the mitogens were diluted in RPMI medium and stored at −70°C. Various concentrations of mitogens were added to the lymphocyte cultures at initiation of culture, and, except for kinetic studies, all cultures were incubated for 4 days.

Inhibition of DNA synthesis. DNA synthesis was inhibited by addition of different concentrations of reagent-grade mitomycin C (Sigma Chemical Co., St. Louis, Mo.) or cytosine arabinoside (The Upjohn Co., Dallas, Tex.) with mitogen to lymphocyte cultures.

Thymidine and amino acid incorporation. Mitogen-stimulated, inhibitor-treated, and control lymphocyte cultures were pulse-labeled for 24 h with 1.0 μCi of [methyl-^{3}H]thymidine (^{3}H-Trd) (specific activity, 48 Ci/mmol; Amersham/Searle, Arlington Heights, Ill.) per ml and for 6 h with 0.5 μCi of L-U-^{14}C-amino acids (specific activity, 55 Ci/mAtom of amino acid mixture; Amersham/Searle) per ml before harvest. Scintillation counts were determined for the fractions precipitable by 10% trichloroacetic acid after adsorption onto 25-mm fiber glass filters (Gelman Instrument Co., Ann Arbor, Mich.).

Interferon production and assay. Supernatant fluids were harvested from treated and untreated cultures at termination of the experiment and stored at −70°C. The samples were assayed for antiviral activity in human amniotic WISH cells by using a 50% plaque reduction method (11) with bovine vesicular stomatitis virus as challenge (40 plaque-forming units/0.1 ml).

RESULTS

Kinetics of mitogen stimulation. Lymphocyte cultures (10^6 lymphocytes in 1.0 ml of RPMI medium) were stimulated with SEA (1.0 μg/ml), PHA-P (10 μg/ml), and ConA (10 μg/ml). ^{3}H-Trd incorporation, ^{14}C-amino acid incorporation,

and immune interferon production were determined over a period of 5 days at 24-h intervals (Fig. 1). Maximal DNA synthesis ([^{3}H]thymidine incorporation) occurred by day 3 for each mitogen. Protein synthesis and interferon production were maximal for all mitogens on either day 3 or day 4. SEA induced about three times more immune interferon (300 versus 100 U/ml) than did PHA-P or ConA. Maximal production of interferon in SEA-treated cultures occurred about 1 day later than in PHA-P- and ConA-treated cultures. The interferon was identified as immune interferon based on its loss of activity at pH 2. There was, in general, a positive correlation among the rates of synthesis of DNA, protein, and interferon for the three mitogens.

Mitogenicity, protein synthesis, and immune interferon production by different concentrations of SEA, PHA-P, and ConA. The amounts of ^{3}H-Trd and ^{14}C-amino acids incorporated and immune interferon produced were determined in lymphocyte cultures incubated for 4 days with different concentrations of SEA (Fig. 2), PHA-P (Fig. 3), and ConA (Fig. 4). ^{3}H-Trd and ^{14}C-amino acid incorporation were maximal or near maximal when cultures were treated with 10^{-5} to 10^2 μg of SEA per ml (Fig. 2). Interferon production by lymphocytes was relatively constant over a slightly narrower concentration range. Thus, SEA gave similar biological activity over a 10^5- to 10^7-fold concentration range. In contrast, the dose response of ^{3}H-Trd and ^{14}C-amino acid incorporation in cultures treated with PHA-P (Fig. 3) and ConA (Fig. 4) was much different than that for SEA. A gradual increase in ^{3}H-Trd and ^{14}C-amino acid incorporation occurred from 0.1 to 10 μg/ml for both PHA-P and ConA, and peak incorporation occurred over relatively narrower ranges (10 to 100 μg/ml and 10 to 30 μg/ml, respectively). ConA at 100 μg/ml inhibited incorporation of ^{3}H-Trd and ^{14}C-amino acids. In general, there was a positive correlation in the rate of synthesis of DNA, protein, and interferon in response to the different doses of the mitogens.

Effect of inhibitors of DNA synthesis on DNA and protein synthesis and interferon production. Various concentrations of mitomycin C and cytosine arabinoside were added to cultures at the same time as SEA, PHA-P, and ConA. The lymphocyte cultures were incubated for 4 days and examined for ^{3}H-Trd and ^{14}C-amino acid incorporation and interferon production. Increasing concentrations of mitomycin C resulted in a corresponding reduction in ^{3}H-Trd and ^{14}C-amino acid incorporation in lymphocytes stimulated by all three mitogens (Fig. 5). For SEA, 1.0 μg of mitomycin C per ml reduced ^{3}H-Trd and ^{14}C-amino acid incorporation to lev-
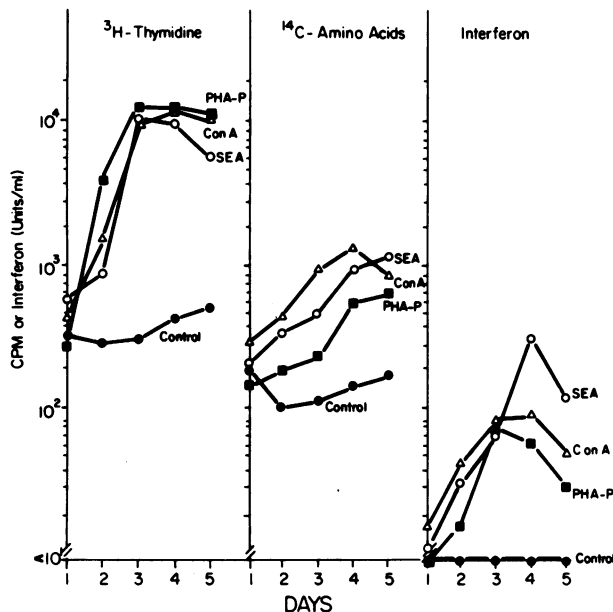
FIG. 1. *Kinetics of [³H]thymidine incorporation, ¹⁴C-amino acid incorporation, and immune interferon production in control human peripheral lymphocyte cultures and in cultures stimulated with SEA (1 µg/ml), PHA-P (10 µg/ml), and ConA (10 µg/ml). Each point represents the mean of lymphocyte cultures from five donors, each done in triplicate.*

els of unstimulated controls. For PHA-P and ConA, 10.0 µg of mitomycin C per ml resulted in the same effect. In the SEA system the immune interferon level decreased from the control (no mitomycin C) level of 300 U/ml to 30 U/ml at 10.0 µg of mitomycin C per ml. In the PHA-P and ConA cultures interferon levels declined at a much slower rate, with the most dramatic decline occurring between 1.0 and 10.0 µg of mitomycin C per ml. Although mitomycin C dramatically reduced the amount of interferon produced by the cultures, reduced levels of interferon were still produced by all three stimulated cultures when DNA and protein syntheses were suppressed to levels of unstimulated cultures. The absence of antiviral activity in fluids harvested from mitomycin C-treated controls is evidence that residual mitomycin C carry-over into the interferon assay was not responsible for the antiviral activity. Another control involved titration of 100 to 300 U of immune interferon in the presence of 0.01 to 10.0 µg of mitomycin C, and this treatment had no effect on the interferon titer. The data suggest that DNA and/or protein synthesis is required for normal immune interferon production, but that reduced interferon production can occur in the absence of detectable DNA and/or protein synthesis in the human lymphocyte system.

Another DNA inhibitor, cytosine arabinoside,

most likely inhibits DNA synthesis by blocking DNA polymerase activity (6, 7). In SEA-stimulated cultures treated with cytosine arabinoside, ³H-Trd and ¹⁴C-amino acid incorporation and interferon production were inhibited in a manner similar to mitomycin C-treated cultures (Fig. 6). The DNA inhibition data of Fig. 5 and 6 suggest a requirement for minimal DNA synthesis over control levels for maximal or near maximal production of immune interferon.

DISCUSSION

SEA has previously been compared with ConA and PHA-P in the murine system and was found to be the most potent suppressor of the in vitro sheep erythrocyte plaque-forming cell response (11). Similarly, SEA was found to be more potent than ConA or PHA-P in the induction of immune interferon, both in terms of mitogen dose requirements and the amount of interferon produced (11). Similar mitogenic effects of SEA were obtained over at least a 1,000-fold concentration range, with the target cell appearing to be associated predominantly with suppressor T-cell activity (10, 11).

In the studies reported here with human peripheral lymphocytes, similar kinetics were observed for SEA, PHA-P, and ConA stimulation in terms of DNA synthesis (³H-Trd uptake), protein synthesis (¹⁴C-amino acid uptake), and
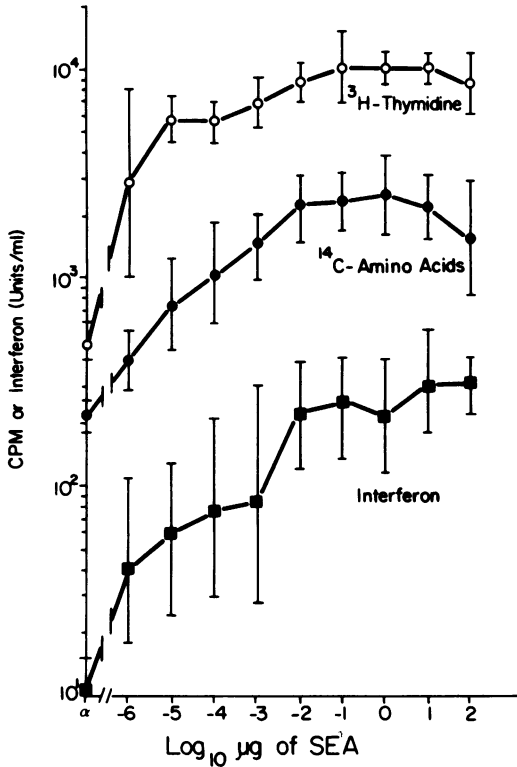
FIG. 2. Mean dose response and 95% confidence limits of [³H]thymidine incorporation, ¹⁴C-amino acid incorporation, and immune interferon production in lymphocyte cultures stimulated with different concentrations of SEA for 20 individuals.

FIG. 3. Mean dose response and 95% confidence limits of [³H]thymidine incorporation, ¹⁴C-amino acid incorporation, and immune interferon production in lymphocyte cultures stimulated with different concentrations of PHA-P for 20 individuals.

immune interferon production (Fig. 1). Maximal responses were obtained in 3 to 4 days. SEA-stimulated cultures produced 300 U of interferon per ml, more than three times the response of PHA-P and ConA. SEA, then, is a better inducer of immune interferon, when compared with PHA-P and ConA.

The kinetics of mitogen-induced immune interferon production in human peripheral lymphocytes corresponded generally to the kinetics of DNA and protein synthesis. Similar findings were reported previously for PHA with respect to DNA and interferon synthesis (5). This is in contrast to the mouse system, in which spleen cells stimulated with SEA, ConA, or PHA-P may yield 55 to 75% of the maximal immune interferon titer in 24 h (11, 17). Thus, in the mouse spleen cell system immune interferon production proceeds at a faster initial rate than does DNA synthesis.

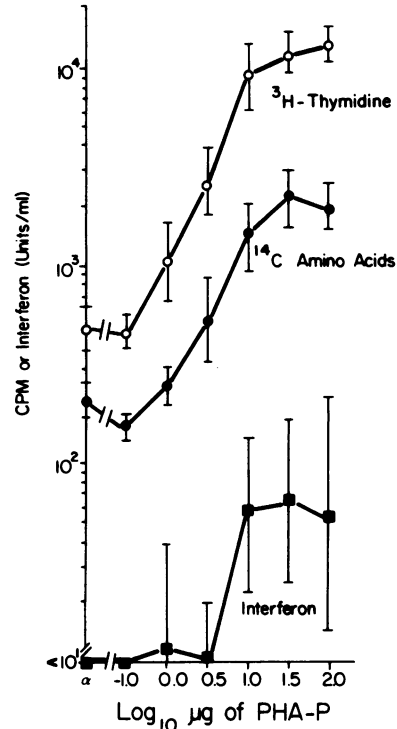Dose response studies also showed a generally positive correlation among DNA and protein synthesis and immune interferon production for the three mitogens (Fig. 2, 3 and 4). The striking observation is the broad concentration range over which SEA showed essentially maximal mitogenic activity. Thus, 10^{-5} to 10^2 µg of SEA per ml resulted in similar uptake of ³H-Trd. This represents a plateau effect of maximal DNA synthesis over a 10^7-fold concentration range. SEA is a single-chained, simple protein with a molecular weight of approximately 28,500 (1, 14). Thus, 10^{-5} µg/ml, the lowest concentration resulting in maximal DNA synthesis by cells, represents 3.5×10^{-13} M SEA. Protein and interferon syntheses were high over slightly narrower concentration ranges. Much higher concentrations of PHA-P and ConA were required for maximal stimulation of cells, and these mitogens showed maximal activity over much narrower concentration ranges than did SEA. Preliminary data show that mitogenesis, interferon induction, and in vitro suppression of the antibody response by SEA are blocked by specific antibody (data not shown).

There are several reasons to suggest that SEA activation of human peripheral lymphocytes was

due to its inherent mitogenic properties rather than to stimulation of sensitized lymphocytes as a result of its antigenic properties. First, the
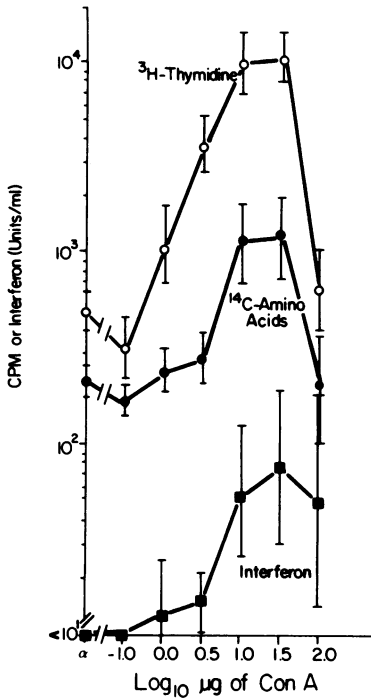

FIG. 4. *Mean dose response and 95% confidence limits of [³H]thymidine incorporation, ¹⁴C-amino acid incorporation, and immune interferon production in lymphocyte cultures stimulated with different concentrations of ConA for 20 individuals.*

mitogenic responses of lymphocytes from 20 individuals to SEA were quite uniform, as indicated by the 95% confidence limits (Fig. 2). These individuals would have had to be similarly sensitized if this were a response to antigen. Second, the kinetics of the mitogenic response with maximal DNA synthesis by day 3 are the same as for PHA-P- and ConA-stimulated cultures. Human peripheral lymphocyte mitogenic responses to activation by SEA as antigen would have been expected to peak at about 4 to 6 days (4). Third, the kinetics and amount of interferon produced are characteristic of T-cell mitogens (5, 9). T-cell mitogens generally induce cultures to produce more interferon than do antigens. Furthermore, the amount of interferon produced by SEA-stimulated cultures was approximately three times greater than that produced by cultures stimulated with PHA-P or ConA. And last, the number of blast cells at the peak of the ³H-Trd uptake was 50 to 60% of the total (data not shown), which is characteristic of mitogen-stimulated cultures (8).

Although the kinetics and dose response data for all the mitogens studied suggested a correlation among DNA, protein, and immune interferon synthesis, it does not mean that these are causal relationships. An examination of mitogen-induced DNA, protein, and immune interferon synthesis in the presence of the DNA inhibitors mitomycin C and cytosine arabinoside was carried out. Immune interferon production was most effectively reduced by concentrations of mitomycin C that reduced DNA and protein synthesis to unstimulated control levels (Fig. 5).
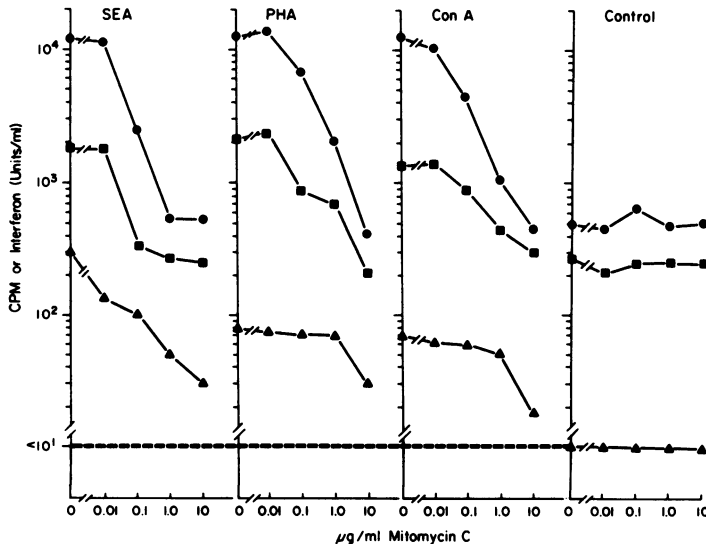

FIG. 5. *Effect of mitomycin C on [³H]thymidine incorporation (●), ¹⁴C-amino acid incorporation (■), and immune interferon production (▲) in control (untreated) lymphocyte cultures and in cultures treated with SEA (1 µg/ml), PHA-P (10 µg/ml), and ConA (10 µg/ml). Each point represents the mean of triplicate cultures.*
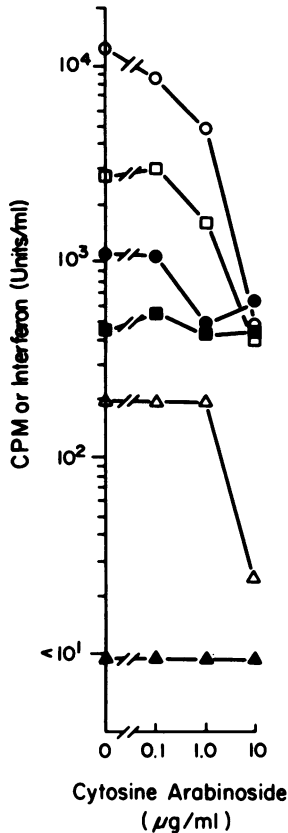
FIG. 6. *Effect of cytosine arabinoside upon [³H]-thymidine incorporation (○), ¹⁴C-amino acid incorporation (□), and immune interferon production (△) in lymphocyte cultures stimulated with SEA (1 μg/ml). Closed symbols represent [³H]thymidine incorporation (●), ¹⁴C-amino acid incorporation (■), and immune interferon production (▲) in control (unstimulated) lymphocyte cultures. Each point represents the mean of triplicate cultures.*

With cytosine arabinoside- and SEA-stimulated cultures a similar pattern was observed (Fig. 6). Complete inhibition of interferon production, however, was not obtained. Thus the data suggest that at least minimal DNA synthesis above control levels may be a prerequisite for normal production of immune interferon in the human peripheral lymphocyte system and that subnormal amounts of immune interferon can be produced in the absence of detectable DNA synthesis above control levels. Studies with PHA-stimulated human lymphocytes that had been irradiated to reduce DNA synthesis and proliferation by >90% resulted in no blockade of interferon production (3). It is not known, however, whether DNA synthesis in this system was suppressed as effectively as we have shown here.

Several lymphocyte functions in the mouse spleen cell system, including immune interferon production and helper and suppressor cell activities, have been dissociated from DNA synthesis and the proliferative response in vitro (19, 20, 22). DNA synthesis in some cases, however, may not have been maintained at or reduced to unstimulated control levels. Furthermore, our results suggest that DNA synthesis and cellular proliferation of human peripheral lymphocytes may be required for maximal and/or expanded biological activity as a function of time.

The data presented here show that SEA is by far the most potent mitogen discovered to date, on a weight and molar basis, for human peripheral lymphocyte stimulation. It is active in concentration ranges observed for hormones (12). The target cell for this stimulation is an E-rosetting T lymphocyte (E. Richie and H. M. Johnson, manuscript in preparation). This is in agreement with previous studies showing that the T lymphocyte is the target cell for SEA in mice (16). Studies are in progress to determine the nature of the membrane receptor on the lymphocyte for SEA as a prerequisite to determining the membrane events that might explain the high biological activity of this substance.

It has been estimated that 45% of all food poisoning is due to staphylococcal enterotoxins (21). The culture filtrates from several strains of *S. aureus* were found to be mitogenic for human peripheral lymphocytes, whereas culture filtrates of *S. albus* were not (18). SEA and other staphylococcal enterotoxins could possibly be responsible for a substantial amount of this mitogenic activity. Because of its high potency and ability to induce interferon and suppressor cell activity, SEA could suppress the immune system after food poisoning or localized *S. aureus* infections.

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