INFECTION AND IMMUNITY, Aug. 1994, p. 3408–3415
0019-9567/94/$04.00+0

Vol. 62, No. 8

# Identification of Staphylococcal Enterotoxin B Sequences Important for Induction of Lymphocyte Proliferation by Using Synthetic Peptide Fragments of the Toxin

MARTI JETT,[1]* ROGER NEILL,[1] CHRISTOPHER WELCH,[1] THOMAS BOYLE,[1] EDWARD BERNTON,[2] DAVID HOOVER,[2] GEORGE LOWELL,[1] ROBERT E. HUNT,[1] SUBROTO CHATTERJEE,[3] AND PETER GEMSKI[1]

*Division of Pathology[1] and Department of Bacterial Diseases,[2] Walter Reed Army Institute of Research, Washington, D.C. 20307-5100, and Department of Pediatrics, Johns Hopkins University, Baltimore, Maryland 21205[3]*

Received 2 December 1993/Returned for modification 25 February 1994/Accepted 26 April 1994

**A series of 13 synthetic peptides, approximately 30 amino acids each, which spanned the entire sequence of staphylococcal enterotoxin B (SEB) were tested to evaluate their effects on T-cell proliferation in a culture system containing elutriated human peripheral blood lymphocytes incubated with a specific ratio of mononuclear cells. Four peptide regions were found to inhibit SEB-induced proliferation; they included sequences 1 to 30 (previously thought to be involved in major histocompatibility complex class II binding), 61 to 92 (sequences which relate to the T-cell receptor site), 93 to 112 (a linear sequence corresponding to the cysteine loop), and 130 to 160 (containing a highly conserved sequence, KKKVTAQEL). Antisera raised to this last peptide were capable of neutralizing SEB-induced proliferation. Antisera raised against the peptides which overlapped this sequence also were somewhat inhibitory. Neutralizing antisera were not produced from any other peptide sequence tested. To determine if any of these effects were nonspecific with regard to SEB-induced proliferation, the peptides were tested for inhibition of phorbol dibutyryl ester-induced proliferation, and only the sequence 93 to 112 (corresponding to the cysteinyl loop region) was consistently inhibitory (40%). Of the regions which displayed inhibition of SEB-induced proliferation, the peptide 130 to 160 inhibited binding of $^{125}$I-SEB to lymphocytes. These data suggest that the residues containing and surrounding the sequence KKKVTAQEL may be critical in the SEB-induced proliferation and may be useful for developing neutralizing antisera to SEB.**

Among the virulence factors associated with the toxinogenic diseases of *Staphylococcus aureus* are the staphylococcal enterotoxins (SEs). These toxins, represented by several serotypes, constitute a group of proteins that share significant sequence homologies in various regions of the toxin molecule (22, 30). These extracellular proteins have been classically recognized as mediating food poisoning characterized by emesis and diarrhea (2).

In addition to this distinguishing enterotoxic property, the SEs possess an added array of biological activities that place them within a functionally and genetically related group of pyrogenic exotoxins whose activities impact several organ systems of susceptible hosts and contribute to clinical syndromes that can culminate in lethal shock (34, 38). These exotoxins share such activities as pyrogenicity (6, 11), enhancement of endotoxic shock (42), induction of immunosuppression (40), induction of a variety of cytokines (1, 14, 39), and mitogenesis of T cells (27, 35). The production of cytokines has been thought to be the cause of lethal shock by these toxins, but it is now apparent that other factors may also be involved (23, 34, 38). Recent studies indicate that SEB is cytotoxic to human proximal kidney cells (8) and exhibits a capacity to alter platelet function and metabolism (46). SEB can increase levels of cyclooxygenase and lipoxygenase metabolites of the arachidonic acid pathway, which may contribute to inflammation, edema, and shock (5, 23, 34).

The nature of the activity of SE on cells of the immune system includes them in a class of molecules designated as superantigens (30). Superantigens are characterized by the ability to serve as ligands simultaneously binding to major histocompatibility complex (MHC) class II receptors on antigen-presenting cells as well as to T-cell receptors (TCR) which express particular Vβ elements. The results of this interaction are T-cell proliferation and cytokine secretion. The repertoire of Vβs activated has a pattern that is characteristic for each SE (10).

Attempts to define the relationship between the structure of SEs and their various biologic activities have focused primarily on the activation of T lymphocytes. These studies indicate that more than one region of the toxin is involved in T-cell activation. Induction of T-cell proliferation was reported for an amino-terminal fragment of SEC (41) and for an amino-terminal synthetic peptide of SEA (36). Another study, however, revealed that T-cell proliferation and pyrogenicity were associated with the carboxy-terminal three-quarters of SEC (4) and a 17-kDa carboxy-terminal fragment of SEB but not an amino-terminal SEB fragment (3). Studies of chimeric molecules from genetic fusions of SEB fragments to protein A indicated that residues 1 to 138 were sufficient to promote T-cell proliferation whereas deletion of the first 30 amino-terminal residues of SEB eliminated activation of at least one Vβ type (7). Deletions of amino-terminal and carboxy-terminal residues of SEA and SEB reduced their mitogenic activity and stability (16, 21, 31).

Other studies have specifically addressed the property of toxin binding to target cells. Synthetic peptides encoding specific amino-terminal and central-region sequences of SEA (12) and a fragment encoding domains in the carboxy-terminal

* Corresponding author. Phone: (202) 576-1490/4490. Fax: (202) 576-0947. Electronic mail address (BITNET): win%"<jett@WRAIR-EMH1.ARMY.MIL>".

half of SEA (17) were all associated with an involvement in binding to the MHC class II receptor. The carboxy-terminal half of SEA, however, did not itself induce T-cell proliferation (17). Substitution mutagenesis of cysteine residues of SEA reduced its mitogenic activity and toxicity in mice but not its binding to MHC class II receptors (13, 14). Analysis of site-directed mutants of SEB reaffirmed the importance of specific amino-terminal and central regions of SEs in MHC class II and Vβ interactions (15, 25). The role of the carboxy-terminal region of SE in the selectivity of interactions with Vβ has been further revealed in studies with hybrids of SEA and SEE (20, 32). The solution of the three-dimensional structure of SEB by X-ray crystallographic analysis has clarified some of these disparate observations by showing that specific amino-terminal and carboxy-terminal residues conformationally encompass a distinct region of the molecule which may represent the TCR-binding site (43).

SEB stimulation of proliferation in mouse spleen cell cultures has been a standard assay for the superantigens (35). However, mice and other rodents are much less responsive to SEB than are primates. SEB causes shock and death in primates at 30 μg/kg, whereas mice can withstand about 200 times that dose (5). For these reasons, we chose to use a human cell system, lymphocytes cultured with monocytes, to test proliferation with a series of overlapping peptides of SEB.

## MATERIALS AND METHODS

**SEB.** SEB, lot 14-30, purified by the method of Schantz et al. (37), was provided by the U.S. Army Research Institute of Infectious Diseases, Frederick, Md. The toxicity of lot 14-30 has been confirmed by recent primate studies (23).

**Synthetic peptides and conjugates of SEB.** On the basis of the published sequence of native SEB (24), a series of 13 peptides (30 amino acids long) were prepared by Peninsula Laboratories, Belmont, Calif. The series contained 15-amino-acid overlaps between adjacent peptide sequences except for the sequence region containing the disulfide loop. Peptides conjugated to keyhole limpet hemocyanin (KLH) were prepared from peptide sequences to which had been added a lauroyl-cysteine residue at the amino-terminal end. A lauroyl residue alone was added to peptide sequences already containing cysteine at the amino-terminal end. Each lauroyl-cysteine peptide was conjugated to KLH via the cysteine residue by the method of Liu et al. (28). A 20-mg portion of peptide was conjugated per 100 mg of KLH. KLH-conjugated peptides were dissolved in water at 4 mg/ml for storage of <2 months at −84°C (solutions were not freeze-thawed). The working concentrations ranged from 0.05 to 10 μg/ml (ca. 2.5 to 500 nM for the peptide). Unconjugated peptides were not readily soluble in water and were first dissolved in dimethyl sulfoxide at 4 mg/ml and stored as described for the KLH-conjugated peptides.

**Polyclonal antibodies to peptides.** Peptides conjugated to KLH were used to hyperimmunize rabbits to prepare polyclonal antisera against the peptide fragments. Rabbits were immunized subcutaneously with 40 μg of peptide-conjugate without adjuvants. Booster immunizations were administered 2 and 7 weeks later. Sera were collected by bleeding from ear veins 2 to 3 weeks after each immunization, and samples were assayed for immunoglobulin G antibody responses to native SEB in an enzyme-linked immunosorbent assay (ELISA) as described previously (33). Titers for binding to SEB were determined from dilutions of sera that gave a half-maximal response in the ELISA. All antisera prepared against peptide-conjugates reacted with native SEB, whereas control sera prepared against KLH did not.

**Cell culture system.** Human peripheral blood mononuclear cells and small lymphocytes were prepared from leukopacks from normal donors by centrifugation over lymphocyte separation medium (Organon Teknika, Durham, N.C.). Monocytes and lymphocytes were then further purified by counterflow centrifugation-elutriation (47) with pyrogen-free Ca- and Mg-free Dulbecco's phosphate-buffered saline (Sigma Chemical Co., St. Louis, Mo.) as the eluant. This procedure resulted in cell preparations of >95% viability with <5% contamination by other, undesired cell types. Generally, cells were used immediately, but in occasional experiments they were stored for 12 to 16 h at 4°C with no impact on the results.

**Proliferation.** Monocyte and lymphocyte preparations were each used separately and combined for SEB-induced proliferation assays. For standard proliferation experiments, lymphocytes were centrifuged from the elutriation buffer and resuspended in culture fluid consisting of RPMI (Biofluids, Gaithersburg, Md.) containing 10% human AB serum (Sigma). Monocytes were left in the elutriation buffer and added to the lymphocyte mixture at a monocyte/lymphocyte ratio of 1:4. The cells were diluted to $2 \times 10^6$ lymphocytes per ml in culture medium, and the cells were plated in 96-well plates (Costar, Cambridge, Mass.) at $0.2 \times 10^6$ lymphocytes per well (100 μl). The volume in the well was increased to 200 μl by the addition of agonist or culture medium or both. The cultures were incubated for 60 h at 37°C in a humidified atmosphere (5% $CO_2$), with 1 μCi of [*methyl*-$^3$H]thymidine added per well for the final 15 h of incubation. The cells were harvested onto fiberglass filter mats by using a cell harvester (Skatron, Sterling, Va.). The mats were air dried, and radioactivity was determined by using a beta counter (LKB/Wallac, Gaithersburg, Md.). Each experiment was performed with eight replicates, and the SEB dose response was determined with each experiment.

**Inhibition of SEB-induced proliferation by peptides or by antiserum to individual peptides. (i) Peptides.** To assess the ability of peptides to inhibit SEB-induced proliferation, peptides or peptide-KLH was added to the wells containing cell cultures. Then, 1 h later, SEB was added at a final concentration of 0.025 μg/ml. The cultures were incubated as described in the previous section.

**(ii) Antisera.** Antisera (50, 100, or 150 μl) were incubated with 0.025 μg of SEB and culture medium for 1 h at 25°C (in a total volume of 300 μl in 24-well plates). At the end of the incubation period, each well was diluted to 1 ml with culture medium, 100 μl was added to eight replicate cell cultures already plated in 96-well plates, the cultures were incubated and harvested, and proliferation was determined as above.

**Phorbol ester-induced proliferation.** Phorbol dibutyryl ester (PDBu) (Sigma) was dissolved in dimethyl sulfoxide at a concentration of 2 mg/ml. It was stored at −84°C in aliquots so that it would not undergo freeze-thaw cycles. The concentrations used in proliferation assays ranged from 2 to 20 ng/ml. For experiments testing peptide inhibition of PDBu-stimulated proliferation, the peptides were incubated with the cell cultures for 1 h and then PDBu, at approximately 50% maximal stimulation, was added to the cultures.

**Inhibition of binding of $^{125}$I-SEB to macrophages or lymphocytes.** SEB was made radioactive with $^{125}$I by using iodogen (Pierce, Rockford, Ill.) (29) and separated from unincorporated $^{125}$I by desalting on a G-25 Sephadex (Pharmacia, Boston, Mass.) column (1 by 30 cm) with deionized water as the eluant. The $^{125}$I-SEB fraction was collected and dried by vacuum centrifugation (Speed-Vac; Savant, Farmingdale,

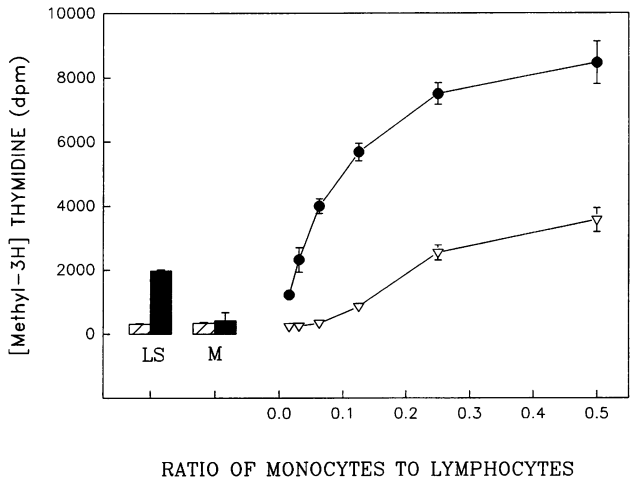


FIG. 1. Determination of proliferation for various ratios of human peripheral blood lymphocytes to monocytes. Lymphocytes ($10^6$) were incubated with 0.01 × $10^6$ to 0.5 × $10^6$ monocytes in the presence of either 0.025 (▽) or 0.2 (●) μg of SEB per ml (in replicates of eight). The bars show the preparations of monocytes (99% purity) or lymphocytes (>99% purity) alone that also were incubated with SEB at 0.025 μg/ml (hatched bar) or 0.2 μg/ml (solid bar). The cultures were incubated for 60 h, with 1 μCi of [*methyl*-$^3$H]thymidine per well added for the final 15 h of incubation. The cells were harvested on fiberglass filter mats, and the radioactivity incorporated was determined as described in Materials and Methods.

N.Y.). Lymphocytes (4 × $10^6$/0.5 ml) or macrophages (2 × $10^6$/0.5 ml) were incubated in a microcentrifuge tube with no additions, with nonradioactive SEB, or with peptide 1–30, 93–112, or 130–160 at concentrations ranging from 10 to 200 μg/ml; 10 min later, $^{125}$I-SEB was added at a concentration one-fifth that of the peptides. The cell suspension was incubated for an additional 1 h at 4°C. The cells were centrifuged at 1,500 × *g* for 10 min, the supernatant solution was decanted, and the cells were resuspended in 200 μl of phosphate-buffered saline (PBS). Lymphocyte separation medium (diluted 50:50 with PBS) was layered under the cell suspension and centrifuged at 15,000 × *g* for 10 min to separate cell-bound from free $^{125}$I-SEB. The supernatant solution was carefully removed (from the top), the microcentrifuge tube tip containing the cell pellet was cut off, and radioactivity was determined in a gamma counter (LKB/Wallac).

## RESULTS

**Induction of proliferation in mixtures of lymphocytes and monocytes by SEB.** Cultures of human peripheral blood lymphocytes and monocytes were stimulated with SEB at various concentrations from 0.025 to 5.0 μg/ml (0.9 to 180 μM). Measurement of incorporation of [*methyl*-$^3$H]thymidine (used as a measure of proliferation) showed that the lowest concentration of SEB which consistently gave an 8- to 10-fold increase above controls was 0.025 μg/ml. Maximal stimulation (40- to 50-fold) was observed with 0.4 to 1 μg of SEB per ml. Higher concentrations of SEB (2 to 10 μg/ml) did not significantly increased thymidine incorporation beyond that seen at 1 μg/ml.

To determine the optimal ratio of lymphocytes to monocytes for maximal response to SEB in the proliferation assay, we varied the number of monocytes while keeping the number of lymphocytes constant (Fig. 1). Two concentrations of SEB were used for this determination. One was designed to achieve maximal stimulation of proliferation (0.2 μg/ml), and the other was in the linear range of stimulation (0.025 μg/ml). A population of lymphocytes (99% purity) was unresponsive to SEB at 0.025 μg/ml but was stimulated approximately fivefold over controls by 0.2 μg of SEB per ml, about fivefold less than the stimulation of the lymphocyte-monocyte mixture (Fig. 1). Monocytes alone (99% purity) showed no increase in [*methyl*-$^3$H]thymidine incorporation at either concentration of SEB. A ratio of monocytes to lymphocytes of 1:4 was observed to give maximal proliferation at either concentration of SEB.

TABLE 1. Structures of synthetic peptide fragments of SEB

| Position (from N terminus)[a] | Amino acid sequence[b] | Structural unit[c] |
|---|---|---|
| 1–30 | ESQPDPKPDELHKSSKFTGLMENMKVLYDD | α1, α2 |
| 21–50 | MENMKVLYDDNHVSAINVKSIDQFLYFDLI | α2, β1 |
| 41–70 | IDQFLYFDLIYSIKDTKLGNYDNVRVEFKN | β2, β3 |
| 61–92 | YDNVRVEFKNKDLADKYKDKYVDVFGANYYYQ | β3, α3, β4 |
| 93–112 | CYFSKKTNDINSHQTDKRKT | Cys loop |
| 101–130 | DINSHQTDKRKTCMYGGVTEHNGNQLDKYR | β5, half β6 |
| 113–144 | CMYGGVTEHNGNQLDKYRSITVRVFEDGKNLL | β5, half β7 |
| 130–160 | RSITVRVFEDGKNLLSFDVQTNKKKVTAQEL | β6, β7, α4 |
| 151–180 | NKKKVTAQELDYLTRHYLVKNKKLYEFNNS | β8, α4, β9 |
| 171–200 | NKKLYEFNNSPYETGYIKFIENENSFWYDM | β8, α4, β9 |
| 191–220 | ENENSFWYDMMPAPGDKFDQSKYLMMYNDN | β9, β10, α5 |

[a] Residue number corresponding to SEB starting at the amino-terminal end of the molecule.

[b] The amino acid sequence of synthetic peptides was based on the nucleotide sequence determined by Jones and Khan (24).

[c] Location of the designated peptide in relation to the α-helices or β-pleated sheets as determined by X-ray crystallography (43).

**Inhibition of proliferation by conjugates of synthetic peptide fragments of SEB.** In an attempt to relate structure to function, we prepared overlapping peptide sequences as shown in Table 1. For the sake of discussion, the area of SEB included in these sequences is identified by their designation in the crystal structure as published by Swaminathan et al. (43).

Of all the peptides that were tested for stimulation of proliferation, only peptide 1–30 showed a two- to threefold increase over control values (data not shown). Similarly, peptides complexed with KLH were also tested to determine if they inhibited or enhanced SEB-induced proliferation. Because the concentration of SEB was crucial to demonstrate inhibition or enhancement, a nonsaturable concentration was selected for these studies (0.025 μg/ml). KLH-conjugated peptides (Fig. 2) were found to be the most effective in the analysis of the inhibition of SEB-induced proliferation. Progressive analyses of peptides representing the amino through carboxyl termini of SEB revealed the following: peptide 1–30, blockage of approximately 55% of SEB-induced proliferation; peptides 21–50 and 41–70, no inhibition observed; peptide 61–92, 50% inhibition; peptide 93–112, 47% inhibition; and the successive two peptides 103–130 and 112–144, no inhibition. Peptide 130–160 was the most inhibitory (62% decreased) of this series of peptides. The remaining three peptides were somewhat inhibitory.

Peptides which showed significant inhibitory activity (Fig. 2) (peptides 1–30, 63–92, 93–112, and 130–160) were next tested at concentrations from 0.1 to 10 μg/ml (Fig. 3). KLH-conjugated peptides 1–30 and 130–160 were both effective at 0.4 μg/ml, and, taking into account the KLH molecule (80% of the weight), it is apparent that these SEB sequences were effective at the equivalent of 20 nM for inhibition of SEB at 0.025 μg/ml

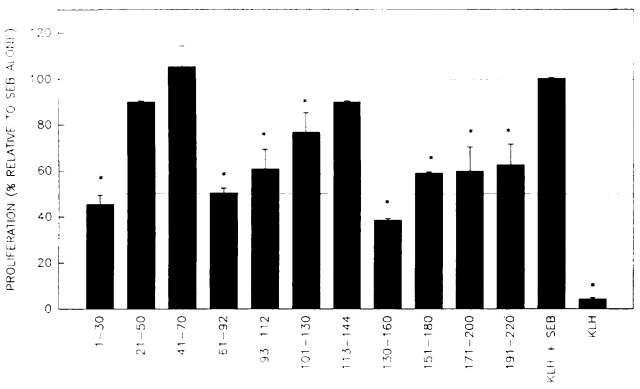


FIG. 2. Inhibition of SEB-induced proliferation by synthetic peptide fragments of SEB conjugated to KLH. Cultures of human peripheral blood monocytes plus lymphocytes (1:4) were incubated with 10 μg of the designated peptide-KLH conjugate per ml for 1 h. SEB was added at 0.025 μg/ml (molar ratio of peptide to SEB, 568:1). The cultures were incubated for 60 h, and proliferation was determined as described in the legend to Fig. 1. These data are the averages of three to five separate experiments with eight replicates each. Student's *t* test was used to compare proliferation in the presence of SEB alone with that in the indicated samples. Asterisks indicate that the differences in the means of the samples are highly significant at the $P = 0.05$ level.

(0.9 nM). Peptide 151–180 was effective at 2 μg/ml (100 μM), and peptides 61–92 and 93–112 were effective at 5 μg/ml (250 nM).

Identical peptide sequences without conjugation to KLH showed a similar pattern of inhibition. The degree of inhibition was not as great as that for the KLH-conjugated peptides under the conditions of our assay (data not shown).

**Antipeptide antiserum inhibition of proliferation.** Antisera to SEB, KLH, and each KLH-conjugated peptide were raised in rabbits. Antisera to peptides reacted with native SEB (see the legend to Fig. 4). In our tests, antisera to SEB were diluted 40-, 20-, and 12.5-fold, preincubated with SEB for 1 h, and added to plated cells. The three dilutions of antisera displayed incremental decreases in SEB-induced proliferation (Fig. 4). In contrast, antisera to KLH displayed no inhibition at even the highest concentration used (Fig. 4). Antisera raised to peptide 130–160 and sequences near it (Fig. 4) were the most effective in blocking SEB-induced proliferation. Up to 59% inhibition with antisera to peptide 130–160, as well as 35 and 47% inhibition with antisera to peptides 112–144 and 151–180, respectively, was achieved. Other antisera (Fig. 4) showed no inhibition at these dilutions, whereas antisera raised against peptides surrounding the cysteinyl loop showed some slight decrease in SEB-induced proliferation (Fig. 4).

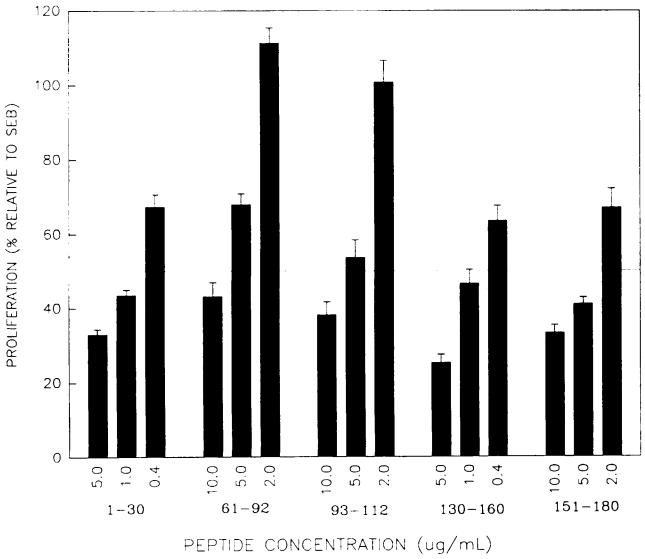


FIG. 3. Dose-response of peptide-KLH conjugate shown to inhibit SEB-stimulated proliferation. The peptide-KLH conjugates shown to be inhibitory (Fig. 2) were incubated at various concentrations with the cell cultures exactly as described in the legend to Fig. 2. The molar ratio of peptide to SEB was 5:1 to 568:1.

**Inhibition of phorbol ester-induced proliferation with peptides.** The peptides were tested with another mitogen, PDBu, to determine if the inhibitory effect they displayed was specific to SEB. PDBu was tested alone at various concentrations in comparison with SEB. SEB and PDBu were seen to be synergistic (Fig. 5, inset). A concentration of PDBu at half-maximal stimulation was chosen, as had been done with SEB (Fig. 5). The only peptide which showed consistent inhibition of PDBu-induced proliferation was peptide 93–112, which contained the linear sequence encompassing the disulfide loop region of SEB (Fig. 6).

**Inhibition of binding of $^{125}$I-SEB by peptide conjugates.** The peptides which had shown some degree of inhibition of SEB-induced proliferation were examined to determine if they would interfere with binding of $^{125}$I-SEB to either monocytes or lymphocytes separately. Direct iodination of peptides was found to present difficulties, which we avoided by using the peptides as inhibitors in the same type of experiment whose results are presented in Fig. 2. We examined binding of $^{125}$I-SEB (4 to 64 μg/ml; 0.296 μCi/μg) and inhibition of binding of $^{125}$I-SEB in the presence of either nonradioactive SEB (fivefold excess) (to determine specific binding) or conjugated peptides 1–30, 93–112, 130–160, and KLH. Each peptide was tested at concentrations ranging from 10 to 400 μg/ml, with $^{125}$I-SEB being approximately the same concentration (in micrograms per milliliter) as the peptide (KLH made up 80% of the conjugate; therefore, the molar ratio of $^{125}$I-SEB to peptide was 2.2:20). $^{125}$I-SEB specific binding to lymphocytes upon preincubation with KLH was decreased to 7% of control values (Fig. 7). Peptide 1–30 showed nearly identical results to those seen with KLH (6.4%). Peptide 93–112 displayed slightly elevated inhibition (11.5%), and peptide 130–160 showed the greatest inhibitory effect (85%), comparable to that seen with nonradioactive SEB (Fig. 7). At the highest concentrations of $^{125}$I-SEB alone, binding to lymphocytes was in the range of 25,000 cpm; 60% of the binding was reversible by using a fivefold excess of nonradioactive SEB. None of the three peptide conjugates significantly altered binding of $^{125}$I-SEB to monocytes (data not shown), in contrast to the inhibition seen with nonradioactive SEB.

## DISCUSSION

Our studies with synthetic peptides have identified a domain of SEB which is involved in regulation of proliferation in cultures of human monocytes and lymphocytes. Peptides 130–160 and 151–180 highlight a region of SEB with the sequence K-K-K-V-T-A-Q-E-L-D (residues 152 to 161), which contains a highly conserved sequence in the pyrogenic exotoxins of *S. aureus* (30). In an accompanying report by Hoffmann et al. (18), this sequence has also been demonstrated to be active in

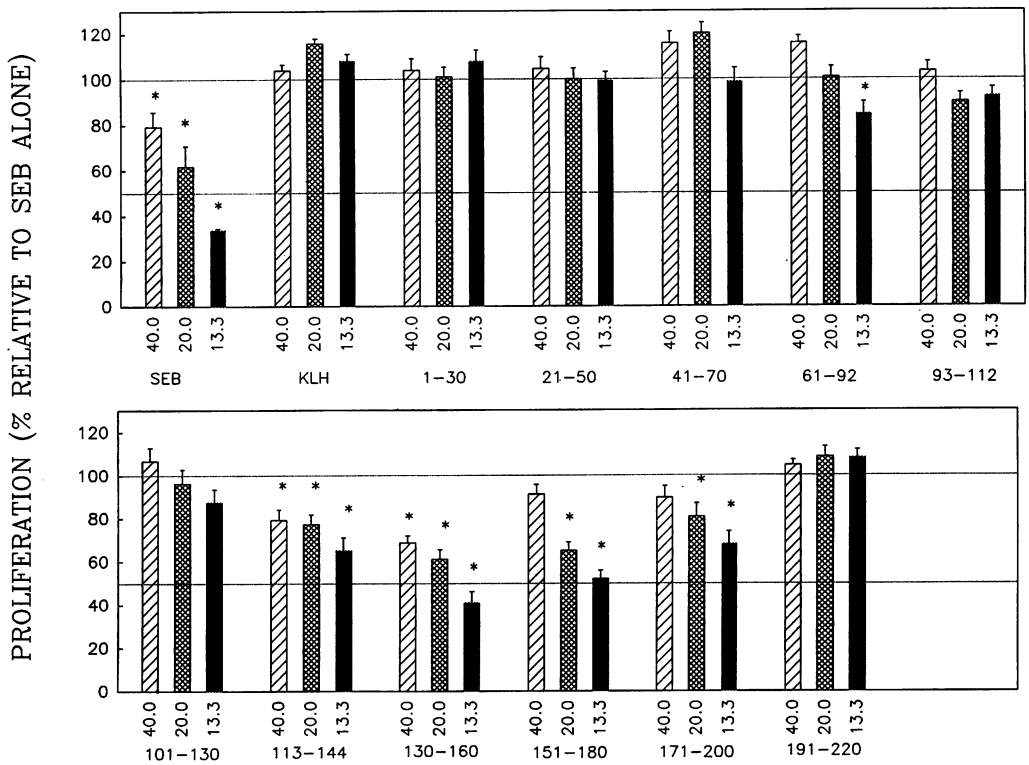


FIG. 4. Preincubation of SEB with antisera raised against peptides to determine possible neutralization of proliferation. Antisera raised to the indicated peptides were each determined to have neutralizing activity to SEB by ELISA. The antiserum dilutions were incubated with SEB for 1 h at 25°C in a volume of 300 μl, diluted to 1 ml, and added to cell cultures. Incubation and determination of proliferation were performed as described in the legend to Fig. 1. Asterisks indicate significant differences as described in the legend to Fig. 2. ELISA titers (per milliliter) of rabbit antisera raised against KLH, KLH-synthetic peptide conjugates, and SEB and tested against native SEB antigen were as follows: anti-KLH, <20; anti-KLH-peptide 1–30, 160; anti-KLH-peptide 21–50, 160; anti-KLH-peptide 41–70, 640; anti-KLH-peptide 61–92, 1,280; anti-KLH-peptide 93–113, 1,280; anti-KLH-peptide 101–130, 1,280; anti-KLH-peptide 113–140, 640; anti-KLH-peptide 130–160, 1,280; anti-KLH-peptide 151–180, 320; anti-KLH-peptide 171–200, 1,280; anti-KLH-peptide 190–220, 2,560; and anti-SEB, 10,240.

a system examining SEC-induced proliferation. Other investigators also have identified regions of another superantigen containing this sequence. Wang et al. (48) recently showed that a synthetic peptide encoding the carboxy-terminal 41 amino acids of the superantigenic PepM5 protein of *Streptococcus pyogenes* blocked PepM5-mediated T-cell proliferation. This peptide contains the sequence motif similar to that described above.

The three-dimensional model based on the crystal structure of SEB (43) would place this sequence at the bottom of a groove in the region which forms a domain functionally associated with both MHC class II binding (D161, R165) and TCR binding (K152, E159, Y162). Our findings that the peptide sequence from 130 to 160 inhibited binding of $^{125}$I-SEB to lymphocytes concurs with this interpretation. This study did not determine selective binding of SEB to T or B lymphocytes. The lack of inhibition of binding of $^{125}$I-SEB to monocytes may be influenced by the absence of lymphocytes in the incubation mixture and may not be completely reflective of the interaction of SEB in its role as a superantigen.

Conjugates of peptides containing sequences from the amino-terminal end of SEB also inhibited cell proliferation induced by native SEB. Peptide conjugate 1–30 inhibited SEB activity by approximately 55% (Fig. 2). Similar inhibition of SEA-induced lymphocyte proliferation (36) was reported for an equivalent amino-terminal peptide of SEA (residues 1 to 27). Moreover, a peptide containing residues 1 to 45 of SEA blocked binding of SEA to MHC class II on Raji cells (12). Analysis of mutants of SEB with site-directed mutations indicated that residues in the amino-terminal end of the toxin (residues 9 to 23) were important in interactions with both MHC class II and TCR (25). In addition, the carboxyl-terminal region of SEB has been associated with activity. Our data indicate significant inhibition of proliferation with different peptide conjugates encompassing residues 190 to 195, for which the three-dimensional position (43) is juxtaposed beside the sequence from 13 to 22. Our peptides 171–200 and 191–220 (near the carboxy terminus) contained those regions (Table 1) and inhibited SEB-induced proliferation by about 40% (Fig. 2). This region may relate more directly to similar inhibition seen with the fragment from 1 to 30. This is further supported by the fact that antisera raised to the peptides 1–30 and 191–220 were completely ineffective in neutralizing SEB-induced proliferation, whereas antiserum raised to peptide 171–200 was somewhat effective. However, the pattern of neutralizing activity, being greatest with peptides 130–160 and 151–180 and gradually losing activity on either side of these regions, suggests that the neutralizing action of peptide 171–200 may relate to the initial portion of the peptide (residue 171). Construction of genetic mutants of the closely related toxins SEA and SEE revealed that carboxy-terminal residues (32), specifically residues 206 and 207 (20), dictate the Vβ specificity of these toxins. Peptide 61–92 was also able to inhibit SEB-induced proliferation. However, the overlapping

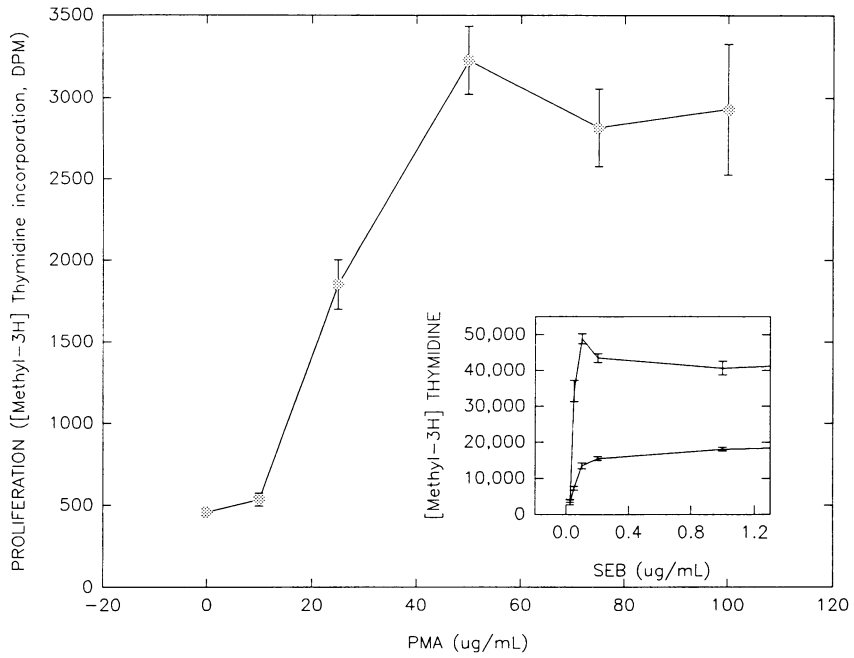


FIG. 5. Dose-response of primary cultures of human lymphocytes and monocytes incubated with PDBu. Cell cultures were incubated with various amounts of PDBu, and proliferation was determined exactly as described in the legend to Fig. 1. Inset shows proliferation of cultures stimulated by SEB alone or in combination with 25 ng of PDBu per ml.

region (peptide 41–70) did not show any inhibition of proliferation, which suggests that the functional region involved amino acid residues between 70 and 90. The structure of the TCR-binding domain identified by the crystallographic studies of Swaminathan et al. (43) suggests that residues 87 to 92 may line the TCR groove. The antiserum to this peptide was not able to neutralize SEB-induced proliferation.

Phorbol esters have been used as costimulatory agents with calcium ionophores (26, 45) and can, by themselves, stimulate the appearance of a surface marker, CD69 (44), which is indicative of protein kinase C activation (45). In this study, we found that PDBu could, by itself, stimulate a low level of proliferation (7-fold above background) whereas SEB stimulated a 40- to 50-fold increase above background. The combination of PDBu and SEB was synergistic, increasing [*methyl*-$^3$H]thymidine incorporation ca. 100-fold. A series of experiments were done to see if any of the peptides were synergistic with PDBu or were able to inhibit PDBu-induced proliferation. Only one peptide (peptide 93–112) was able to inhibit both SEB- and PDBu-stimulated proliferation. Antisera raised to that peptide were ineffective in preventing SEB-induced proliferation. In SEB, these residues make up the

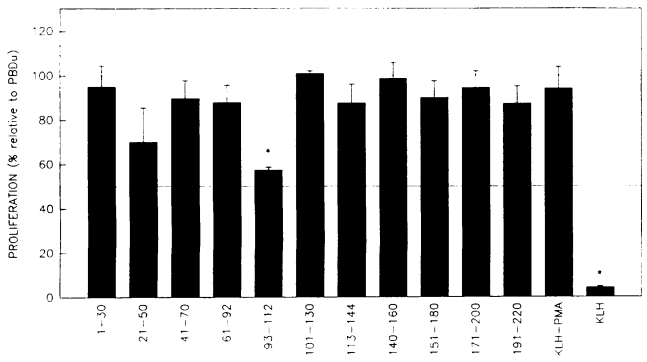


FIG. 6. Determination of inhibition of PDBu-stimulated proliferation by peptide fragments of SEB. Peptides were incubated with cell cultures for 1 h, and PDBu was added at 25 ng/ml. Cultures were incubated and harvested, and the significant differences were identified exactly as described in the legend to Fig. 2.

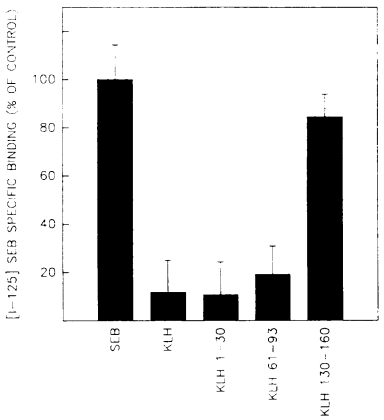


FIG. 7. Test of peptide inhibition of binding of $^{125}$I-SEB to human lymphocytes. Conjugated peptides (10 to 200 μg/ml) were incubated with $4 \times 10^6$ lymphocytes per ml for 10 min, after which time $^{125}$I-SEB was added to the cultures at 1/10 the molar concentration of the peptide (to mimic the conditions described in the legend to Fig. 3); KLH inhibition of binding was determined at the microgram-per-milliliter concentrations used for the peptide conjugates. Binding of $^{125}$I-SEB in the presence of a fivefold excess of nonradioactive SEB showed the specific binding to lymphocytes. After 1 h, the radioactivity bound to the cells was determined as described in Materials and Methods.

disulfide loop region (the synthetic peptide did not have a disulfide bridge). In human proximal tubule kidney cell cultures, this peptide was quite cytotoxic (9). These findings lead us to suspect that, rather than blocking SEB-induced proliferation by occupying a receptor site, this peptide could have been directly cytotoxic to the cells under the conditions of this proliferation assay. Hovde et al. (19) have also found biological activity associated with this region of SEC.

There are numerous explanations for the ineffectiveness of many antisera in blocking the SEB proliferative response. Antibodies that bind to functional domains of SEB may not always result in neutralization of toxin activity. In addition, antibodies to conformational epitopes of native SEB may not be present in these antisera, since it is likely that the conformation of the peptide may not sufficiently resemble the three-dimensional structure associated with those regions of the native molecule. Alternatively, one could argue that antibody binding to SEB could alter the conformation of SEB and cause inhibition of its activity. Antiserum raised against peptide 130–160 was certainly the most effective at preventing SEB-stimulated proliferation. Peptide 130–160, which is immunogenic for neutralizing antisera, may present opportunities for use as a possible vaccine candidate.

## ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of James Wootres, Boris Ionin, Hasib Kazim, Gregory Greene, and Charles Opperman.

## REFERENCES

1. **Bean, A. G. D., R. A. Freiberg, S. Andrade, S. Menon, and A. Zlotnik.** 1993. Interleukin 10 protects mice against staphylococcal enterotoxin B-induced lethal shock. Infect. Immun. **61**:4937–4939.
2. **Bergdoll, M. S.** 1970. Enterotoxins, p. 265–326. *In* S. J. Ajl, T. C. Montie, and S. Kadis (ed.), Microbial toxins. Academic Press, Inc., New York.
3. **Binek, M., J. R. Newcomb, C. M. Rogers, and T. J. Rogers.** 1992. Localisation of the mitogenic epitope of staphylococcal enterotoxin B. J. Med. Microbiol. **36**:156–163.
4. **Bohach, G. A., J. P. Handley, and P. M. Schlievert.** 1989. Biological and immunological properties of the carboxyl terminus of staphylococcal enterotoxin C1. Infect. Immun. **57**:23–28.
5. **Boyle, T., V. Lancaster, R. Hunt, P. Gemski, and M. Jett.** 1994. Method for simultaneous isolation and quantitation of platelet activating factor and multiple arachidonate metabolites from small samples: analysis of effects of Staphylococcus aureus enterotoxin B in mice. Anal. Biochem. **216**:373–382.
6. **Brunson, K. W., and D. W. Watson.** 1974. Pyrogenic specificity of streptococcal exotoxins, staphylococcal enterotoxin, and gram-negative endotoxin. Infect. Immun. **10**:347–351.
7. **Buelow, R., R. E. O'Hehir, R. Schreifels, T. J. Kummerehl, G. Riley, and J. R. Lamb.** 1992. Localization of the immunologic activity in the superantigen staphylococcal enterotoxin B using truncated recombinant fusion proteins. J. Immunol. **148**:1–6.
8. **Chatterjee, S., and M. Jett.** 1992. Glycosphingolipids: the putative receptor for staphylococcus aureus enterotoxin-B in human kidney proximal tubular cells. Mol. Cell. Biochem. **113**:25–31.
9. **Chatterjee, S., M. Jett, R. Neill, and P. Gemski.** Unpublished data.
10. **Choi, Y., B. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler.** 1989. Interaction of Staphylococcus aureus toxin "superantigens" with human T cells. Proc. Natl. Acad. Sci. USA **86**:8941–8945.
11. **Clark, W. G., and H. L. Borison.** 1963. Pyrogenic effect of purified staphylococcal enterotoxin. J. Pharmacol. Exp. Ther. **142**:237–241.
12. **Griggs, N. D., C. H. Pontzer, M. A. Jarpe, and H. M. Johnson.** 1992. Mapping of multiple binding domains of the superantigen staphylococcal enterotoxin A for HLA. J. Immunol. **148**:2516–2521.
13. **Grossman, D., R. G. Cook, J. T. Sparrow, J. A. Mollick, and R. R. Rich.** 1990. Dissociation of the stimulatory activities of staphylococcal enterotoxins for T cells and monocytes. J. Exp. Med. **172**:1831–1841.
14. **Grossman, D., J. G. Lamphear, J. A. Mollick, M. J. Betley, and R. R. Rich.** 1992. Dual roles for class II major histocompatibility complex molecules in staphylococcal enterotoxin-induced cytokine production and in vivo toxicity. Infect. Immun. **60**:5190–5196.
15. **Harris, T. O., D. Grossman, J. W. Kappler, P. Marrack, R. R. Rich, and M. J. Betley.** 1993. Lack of complete correlation between emetic and T-cell-stimulatory activities of staphylococcal enterotoxins. Infect. Immun. **61**:3175–3183.
16. **Harris, T. O., W. O. Hufnagle, and M. J. Betley.** 1993. Staphylococcal enterotoxin type A internal deletion mutants: serological activity and induction of T-cell proliferation. Infect. Immun. **61**:2059–2068.
17. **Hedlund, G., M. Dohlsten, T. Herrmann, G. Buell, P. A. Lando, S. Segrén, J. Schrimsher, H. R. MacDonald, H. O. Sjögren, and T. Kalland.** 1991. A recombinant C-terminal fragment of a staphylococcal enterotoxin A binds to human MHC class II products, but does not activate T cells. J. Immunol. **147**:4082–4085.
18. **Hoffmann, M. L., L. M. Jablonski, K. K. Crum, S. P. Hackett, Y.-I. Chi, C. V. Stauffacher, D. L. Stevens, and G. A. Bohach.** 1994. Predictions of T-cell receptor- and major histocompatibility complex-binding sites on staphylococcal enterotoxin C1. Infect. Immun. **62**:3396–3407.
19. **Hovde, C. J., J. C. Marr, M. L. Hoffman, S. P. Hackett, K. K. Crum, D. L. Stevens, and G. A. Bohach.** Investigation of the role of the disulfide bond in activity and structure of staphylococcal enterotoxin C1. Submitted for publication.
20. **Hudson, K. R., H. Robinson, and J. D. Fraser.** 1993. Two adjacent residues in staphylococcal enterotoxins A and E determine T cell receptor VB specificity. J. Exp. Med. **177**:175–184.
21. **Hufnagle, W. O., M. T. Tremaine, and M. J. Betley.** 1991. The carboxyl-terminal region of staphylococcal enterotoxin type A is required for a fully active molecule. Infect. Immun. **59**:2126–2134.
22. **Iandolo, J. J.** 1989. Genetic analysis of extracellular toxins of *Staphylococcus aureus*. Annu. Rev. Microbiol. **43**:375–402.
23. **Jett, M., W. Brinkley, R. Neill, P. Gemski, and R. Hunt.** 1990. *Staphylococcus aureus* enterotoxin B challenge of monkeys: correlation of plasma levels of arachidonic acid cascade products with occurrence of illness. Infect. Immun. **58**:3494–3499.
24. **Jones, C. L., and S. A. Khan.** 1986. Nucleotide sequence of the enterotoxin B gene from *Staphylococcus aureus*. J. Bacteriol. **166**:29–33.
25. **Kappler, J. W., A. Herman, J. Clements, and P. Marrack.** 1992. Mutations defining functional regions of the superantigen staphylococcal enterotoxin B. J. Exp. Med. **175**:387–396.
26. **Kumagai, N., S. H. Benedict, G. B. Mills, and E. W. Gelfand.** 1987. Requirements for the simultaneous presence of phorbol esters and calcium ionophores in the expression of human T lymphocyte proliferation-related genes. J. Immunol. **139**:1393–1399.
27. **Langford, M. P., G. J. Stanton, and H. M. Johnson.** 1978. Biological effects of staphylococcal enterotoxin A on human peripheral lymphocytes. Infect. Immun. **22**:62–68.
28. **Liu, F.-T., M. Zinnecker, T. Hamaoka, and D. H. Katz.** 1979. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of such conjugates. Biochemistry **18**:690–697.
29. **Markwell, M. A., and C. F. Fox.** 1978. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3alpha,6alpha-diphenylglycoluril. Biochemistry **17**:4807–4817.
30. **Marrack, P., and J. Kappler.** 1990. The staphylococcal enterotoxins and their relatives. Science **248**:705–711.
31. **Metzroth, B., T. Marx, M. Linnig, and B. Fleischer.** 1993. Concomitant loss of conformation and superantigenic activity of staphylococcal enterotoxin B deletion mutant proteins. Infect. Immun. **61**:2445–2452.
32. **Mollick, J. A., R. L. McMasters, D. Grossman, and R. R. Rich.** 1993. Localization of a site on bacterial superantigens that determines T cell receptor B chain specificity. J. Exp. Med. **177**:283–293.
33. **Neill, R. J., G. R. Fanning, F. Delahoz, R. Wolff, and P. Gemski.**

1990. Oligonucleotide probes for detection and differentiation of *Staphylococcus aureus* strains containing genes for enterotoxins A, B, and C and toxic shock syndrome toxin 1. J. Clin. Microbiol. **28:**1514–1518.

34. **Parsonnet, J.** 1989. Mediators/pathogenesis. Mediators in the pathogenesis of toxic shock syndrome: overview. Rev. Infect. Dis. **11:**S263.
35. **Peavy, D. L., W. H. Adler, and R. T. Smith.** 1970. The mitogenic effects of endotoxin and staphylococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes. J. Immunol. **105:**1453.
36. **Pontzer, C. H., J. K. Russell, and H. M. Johnson.** 1989. Localization of an immune functional site on staphylococcal enterotoxin A using the synthetic peptide approach. J. Immunol. **143:**280–284.
37. **Schantz, E. J., W. G. Roessler, J. Wagman, L. Spero, D. A. Dunnery, and M. S. Bergdoll.** 1965. Purification of staphylococcal enterotoxin B. Biochemistry **4:**1011–1016.
38. **Schlievert, P. M.** 1993. Role of superantigens in human disease. J. Infect. Dis. **167:**997–1002.
39. **Scholl, P. R., N. Trede, T. A. Chatila, and R. S. Geha.** 1993. Role of protein tyrosine phosphorylation in monokine induction by the staphylococcal superantigen toxin shock syndrome toxin-1. J. Immunol. **148:**2237–2241.
40. **Smith, B. G., and H. M. Johnson.** 1975. The effect of staphylococcal enterotoxins on the primary in vitro immune response. J. Immunol. **115:**575.
41. **Spero, L., and B. A. Morlock.** 1978. Biological activities of the peptides of staphylococcal enterotoxin C formed by limited tryptic hydrolysis. J. Biol. Chem. **263:**8787–8791.
42. **Sugiyama, H., E. M. McKissic, Jr., M. S. Bergdoll, and B. Heller.** 1964. Enhancement of bacterial endotoxin lethality by staphylococcal enterotoxin. J. Infect. Dis. **114:**111–118.
43. **Swaminathan, S., W. Furey, J. Pletcher, and M. Sax.** 1992. Crystal structure of staphylococcal enterotoxin B, a superantigen. Nature (London) **359:**801–806.
44. **Testi, R., J. H. Phillips, and L. L. Lanier.** 1989. T cell activation via Leu-23 (CD69). J. Immunol. **143:**1123–1128.
45. **Testi, R., J. H. Phillips, and L. L. Lanier.** 1989. Lev 23 induction as an early marker of functional CD3/T cell antigen receptor triggering. J. Immunol. **142:**1854–1860.
46. **Tran, U., T. Boyle, and M. Jett.** 1993. Staphylococcal enterotoxin B (SEB) inhibition of aggregation and adhesion in human thrombocytes: activation of protein kinase C and arachidonate metabolism. FASEB J. **7:**A1234.
47. **Wahl, L. M., I. M. Katoona, R. L. Wilder, C. C. Winter, B. Haraoui, I. Scher, and S. Wahl.** 1984. Isolation of human mononuclear cell subsets by counter flow centrifugal elutriation (CCE). I. Characterization of B-lymphocytes, T-lymphocytes, and monocyte-enriched fractions by flow cytometric analysis. Cell. Immunol. **85:**373.
48. **Wang, B., P. M. Schlievert, A. O. Gaber, and M. Kotb.** 1993. Localization of an immunologically functional region of the streptococcal superantigen pepsin-extracted fragment of type 5 M protein. J. Immunol. **151:**1419–1429.