INFECTION AND IMMUNITY, Sept. 1994, p. 3907–3915
0019-9567/94/$04.00+0

Vol. 62, No. 9

Binding and Activation of Major Histocompatibility Complex Class II-Deficient Macrophages by Staphylococcal Exotoxins†

ALISON A. BEHARKA,[1] JASON W. ARMSTRONG,[1] JOHN J. IANDOLO,[2] AND STEPHEN K. CHAPES[1,2]*

Division of Biology[1] and Department of Pathology and Microbiology,[2] Kansas State University, Manhattan, Kansas

Received 28 April 1994/Returned for modification 1 June 1994/Accepted 30 June 1994

Macrophages from C2D transgenic mice deficient in the expression of major histocompatibility complex (MHC) class II proteins were used to identify binding sites for superantigens distinct from the MHC class II molecule. Iodinated staphylococcal enterotoxins A and B (SEA and SEB) and exfoliative toxins A and B (ETA and ETB) bound to C2D macrophages in a concentration-dependent and competitive manner. All four toxins increased F-actin concentration within 30 s of their addition to C2D macrophages, indicating that signal transduction occurred in response to toxin in the absence of class II MHC. Furthermore, ETA, ETB, SEA, and, to a lesser extent, SEB induced C2D macrophages to produce interleukin 6. Several molecular species on C2D macrophages with molecular masses of 140, 97, 61, 52, 43, and 37 kDa bound SEA in immunoprecipitation experiments. These data indicate the presence of novel, functionally active toxin binding sites on murine macrophages distinct from MHC class II molecules.

The gram-positive bacterium *Staphylococcus aureus* secretes up to 30 different exotoxins into its environment. Among these are staphylococcal enterotoxin A (SEA), the major causative agent of food poisoning, staphylococcal enterotoxin B (SEB), toxic shock syndrome toxin 1, and exfoliative toxin A (ETA) and exfoliative toxin B (ETB), which induce scalded skin syndrome (3, 26, 27). These staphylococcal exotoxins are potent T-cell stimulants and have been called superantigens (44). Enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 activate human, rat, and mouse macrophages to secrete tumor necrosis factor (TNF), NO^-, interleukin 1 (IL-1), and IL-6 (2, 13–15, 28, 35, 36). Furthermore, macrophages become cytolytic in the presence of these exotoxins and gamma interferon (13, 15).

The major histocompatibility complex class II (MHC II) molecule is a high-affinity receptor for staphylococcal superantigens. Immunoprecipitation and autoradiography studies demonstrated a direct interaction between staphylococcal exotoxins and the MHC II molecule (16, 33, 39). This interaction is unusual because unprocessed toxin binds to the nonpolymorphic regions of the MHC II molecule, away from the traditional antigen presenting site (10, 23, 38). The toxin-MHC molecule interaction occurs because toxins do not require processing and because the interaction satisfies the simple thermodynamic rules of receptor-ligand interactions. Therefore, the possibility exists that other molecules may be present on cells that have similar structural properties that allow interaction with toxin.

A superantigen binding site, distinct from the MHC II molecule, has been inferred from several studies (4, 24, 34, 43). In particular, in our laboratory, Scatchard analysis of competitive binding data from experiments using B6MP102 bone marrow-derived macrophages indicated the presence of two staphylococcal exotoxin binding receptors. However, in spite of this compelling evidence for the presence of non-MHC II superantigen binding receptors, little is known about these molecules.

Transgenic mice deficient in MHC II molecules (GenPharm International, Mountain View, Calif.) provide an excellent way to study non-MHC II toxin-binding molecules. C2D mice lack cell surface expression of MHC II molecules because of a null mutation in the MHC II Aβ gene (18). Therefore, experiments using cells from C2D mice are not subject to the criticisms made of other methods used to study alternate toxin-binding molecules. There are no concerns over whether the monoclonal antibodies used to inhibit toxin binding completely block the MHC II binding receptor or over whether MHC II molecules are present on cells at concentrations below the sensitivity of the detection assays. These experiments are also not subject to criticisms that MHC II-negative tumor cell lines are not representative of normal cells. Therefore, we used C2D transgenic mice to investigate whether staphylococcal superantigens bind and activate macrophages through a receptor distinct from the MHC II molecules.

MATERIALS AND METHODS

Mice. Transgenic mice, negative for MHC class I (MHC I) (C1D, H-2^b mice) or MHC II (C2D, H-2^b mice), were purchased from GenPharm, Int. The C1D mice were used as a syngeneic, MHC II-positive control that had undergone a genetic manipulation at a locus distinct from MHC II. We confirmed earlier findings (18) that C1D and C2D mice lacked MHC I and II molecules, respectively, by using flow cytometric analysis of thymocytes or spleen cells (1). Additionally, same-age C57BL/6 (B6, H-2^b) mice (Jackson Laboratory, Bar Harbor, Maine) were used as unmanipulated, syngeneic, MHC II-positive controls.

Cells. C1D, C2D, and B6 murine, peritoneal macrophages were obtained by peritoneal lavage as previously described (15). Bone marrow cells were recovered from long bones and were grown into macrophage monolayers on 60- or 100-mm-diameter tissue culture dishes in Dulbecco's modified Eagle medium (DMEM) (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum, 0.3% L-glutamine, and 15% LM-929 conditioned cell supernatant (as a source of colony-stimulating factor 1). Bone marrow cells were allowed to remain in culture for a minimum of 2 weeks before use to

* Corresponding author. Mailing address: Division of Biology, Ackert Hall, Kansas State University, Manhattan, KS 66506-4901.

† Kansas Agricultural Experiment Station Publication 94-162-J.

allow for the differentiation into macrophages. The TNF-sensitive cell line LM-929 (American Type Culture Collection CCL 1.2) was used as a source of colony-stimulating factor 1 and to detect TNF. LM-929 cells were passaged 2 to 3 times weekly in antibiotic-free DMEM supplemented with 2% fetal bovine serum. IL-6 was quantified with the IL-6-dependent, murine B-cell hybridoma subclone B9 (15). It was cultured in DMEM supplemented with 50 μM 2-mercaptoethanol, 5% fetal bovine serum, and 10 pg of recombinant IL-6.

Toxin. Purified preparations of SEA and SEB were obtained from Toxin Technologies (Sarasota, Fla.). SEA and SEB are a minimum of 96% pure as determined by the supplier. Alternate preparations of SEB were obtained from the U.S. Army Medical Research and Development command (Ft. Detrick, Md.). Furthermore, every new preparation of endotoxin was run through a sodium dodecyl sulfate (SDS) gel and was stained with silver (Bio-Rad Silver Stain Plus, with a sensitivity of approximately 10 ng) to check for impurities. SEA consistently stained as a single band, and both SEB preparations stained as a doublet with a molecular mass of 25 kDa. ETA and ETB were prepared in our laboratory by using strains of *Staphylococcus aureus* and the purification procedure previously described (15). ETA and ETB preparations gave single bands on gels stained with silver. The enterotoxins had ≤0.4 ng of endotoxin-like reactive material per μg of toxin, and the exfoliative toxins had less than 2.0 ng per μg, as determined by the Limulus amebocyte lysate assay (Sigma, St. Louis, Mo.). Our assay was sensitive to a concentration of 0.02 ng/ml.

Saturation and competitive binding assays and determination of K_d. Exotoxins were iodinated with ^{125}I (NEN/Dupont, Boston, Mass.) by using the chloramine-T method. Iodination of toxins achieved a level of at least 8 μCi/μg. The labeled toxins were aliquoted, stored at −86°C, and used within 2 weeks of iodination. To determine if toxin bound cells in a saturable manner, 10^6 peritoneal macrophages were added per well of a round-bottomed 96-well plate and were incubated with 1 μg of ^{125}I-labeled toxin and 0-100 μg of unlabeled toxin. Specific binding was determined by subtracting the signal obtained from ^{125}I-labeled toxin binding in the presence of a 100-fold excess of unlabeled toxin from the signal obtained with labeled toxin alone.

For competitive binding studies, 2×10^5 to 1×10^6 peritoneal or bone marrow-derived macrophages were added per well of a round-bottomed 96-well plate and were incubated on ice with 0 to 64 μg of unlabeled toxin. Between 0.5 μg and 3 μg of ^{125}I-labeled exotoxin was added. The assay was incubated a minimum of 60 min on ice in a total volume of 200 μl of medium which allowed 80 to 100% of the maximum toxin binding possible under these conditions, as determined by previous experiments in our laboratory. Assays were conducted at 4°C to avoid activation and cell attachment to the wells of the microtiter plate. Cells were pelleted by centrifugation at $325 \times g$, and the supernatant was removed and counted on a gamma counter. Pelleted cells were washed four times, resuspended in 200 μl of medium, and counted. All treatments were done in triplicate, and the experiment was repeated a minimum of three times. Dissociation constants (K_ds) and receptor numbers were determined by Scatchard analysis because of the reversible nature of the toxin-cell interaction (12).

Determination of F-actin. We used a modification of the methodology of Howard and Meyer (25) to measure the toxin-induced change in polymerized actin in macrophages. Macrophages (10^6 cells per 100 μl of phosphate-buffered saline [PBS]) were suspended in sterile, 1.5-ml Eppendorf tubes. Ten microliters of toxin (10 μg/ml) or, as a positive control, F-Met-Leu-Phe (10^{-6} M, Sigma) (25) was added to cells and incubated for 30 s. The reaction was stopped by adding 10 μl of phosphate-buffered formalin (final concentration, 3.7%) to each treatment group, including the controls, followed by incubation at 37°C for 5 min. Five μl of the fluorescent, actin-binding compound NBD [*N*-(7-nitrobenz-2 oxa-1, 3 diazol 4)]-phallacidin (Molecular Probes, Eugene, Ore.) (according to the manufacturer, having an excitation maximum of 465 nM, an emission maximum of 530 nM, and a K_d of 18 nM for actin and reconstituted according to the manufacturer's directions) was added to each preparation and was incubated for 10 min at 37°C. The cells were washed twice in PBS, resuspended in PBS, and analyzed on a FACScan flow cytometer (Becton Dickinson, Sunnyvale, Calif.) as previously described (25).

Macrophage stimulation. Macrophages were plated at a density of 10^6 cells per 60-mm-diameter tissue culture plate and were allowed to adhere 1 h, and then the medium was removed. Three milliliters of DMEM containing the appropriate stimulus (10 μg of toxin per ml or 12.5 μg of lipopolysaccharide per ml [used as a positive control]) or medium alone (control) was added for 16 h. After this time, the supernatants were collected, clarified by centrifugation, aliquoted, and used immediately or stored at −86°C until assayed for cytokine secretion.

Cytokine and nitrite quantitation. The macrophage supernatants were assayed for IL-6 content by using the B9 bioassay (21). The B9 cells were washed three times in IL-6-free RPMI medium to remove residual IL-6. Four thousand B9 cells were added to serially diluted, triplicate samples of culture supernatant and were allowed to incubate for 3 days at 37°C. Cell death was quantified by using MTT (1-[4,5-dimethylthiazol-2-Y1]-2,5-diphenyltetrazolium bromide). Fifty μl of MTT (2 mg/ml) was added to each assay well and was incubated for 3 h at 37°C. The medium was removed, and 150 μl of Iso-PBS (100 ml of isopropyl alcohol, 40 ml of 5 N HCl, and 50 ml of PBS) was added to dissolve crystals. A microtiter plate reader (Cambridge Technologies, Watertown, Mass.) was used to read A_{570}.

TNF was quantified as described previously (15) except that MTT was used (see description above). Nitrite concentration was determined by use of the Griess reagent (1% sulfanilamide, 0.1% naphylethylene diamine dihydrochloride, and 2% H_3PO_4) as previously described (41). Cytokine and nitrite quantification was based on linear regression of standard curves of recombinant TNF, recombinant IL-6, or $NaNO_2$. Recombinant murine TNF was obtained from Genzyme (Cambridge, Mass.), and recombinant murine IL-6 was obtained from R and D Systems (Minneapolis, Minn.).

Immunoprecipitation. B6MP102 cells and C2D peritoneal macrophages (10^8) were surface labeled with ^{125}I (NEN/Dupont, Boston, Mass.) with lactoperoxidase (Calbiochem) and were incubated with or without 50 μg of unlabeled SEA per ml for 2 h at 4°C. Washed cells were lysed in 1 ml of ice-cold lysis buffer (2% Nonidet P-40, 15 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, and aprotinin). Lysates were precleared for 1 h at 4°C with normal rabbit serum followed by the addition of fixed *S. aureus* bacteria (Pansorbin; Calbiochem). Aliquots were immunoprecipitated with rabbit anti-SEA serum and were immobilized on Pansorbin. Precipitates were washed three times with ice-cold lysis buffer and then boiled in SDS sample buffer, centrifuged, and run on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel. ^{125}I-labeled receptors that bound toxin were imaged by exposing dried gels to X-ray film.

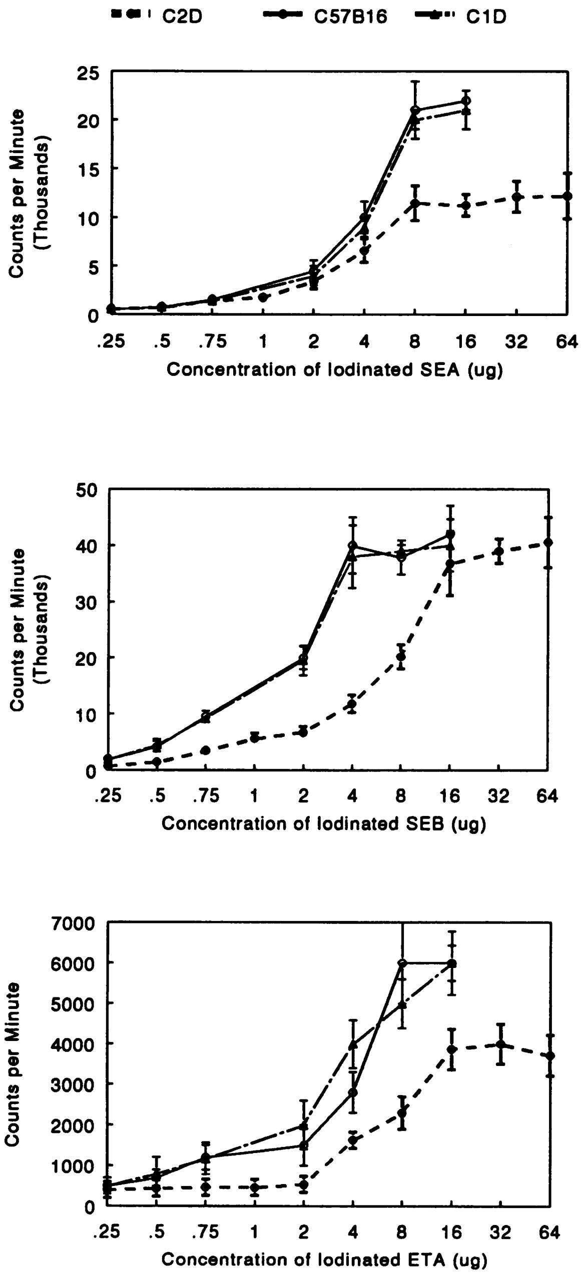

FIG. 1. Dose response curves demonstrating specific and saturable binding of ^{125}I-SEA, ^{125}I-SEB, and ^{125}I-ETA to 10^6 C2D, C1D, or C57BL/6 peritoneal macrophages. Specific binding is the difference between the signal obtained with labeled toxin alone and the signal obtained in the presence of a 100-fold excess of unlabeled toxin. These graphs represent the results of at least three experiments.

RESULTS

Macrophage binding by staphylococcal exotoxins. We determined if exotoxins could bind to non-MHC II receptors on C2D macrophages. Additionally, we calculated the affinity of the interaction between superantigen and non-MHC II receptors. Iodinated SEA, SEB, ETA (Fig. 1), and ETB (data not shown) specifically bound to MHC II-positive and -negative peritoneal and bone marrow-derived macrophages in a concentration-dependent manner, and the binding was saturable for SEA, SEB, and ETA (Fig. 1) (ETB was not tested in these assays). The kinetics of toxin binding to C2D peritoneal macrophages was similar to that to control macrophages, but higher toxin concentrations were necessary to achieve binding comparable to that of MHC II-positive controls (Fig. 1). Iodinated SEA and SEB also bound C2D cells isolated from lymph nodes, in a similar concentration-dependent way (data not shown).

Competitive binding assays, in which 0.5 to 3 μg of iodinated SEA or SEB was inhibited with increasing concentrations of cold SEA or SEB, respectively, indicated that competition occurred for a limited number of binding sites on both MHC II-positive (Fig. 2) and -negative cells. However, the kinetics of the inhibition differed for the MHC II-positive and -negative cells. For the MHC II-positive cells, it usually took less than 6 μg of cold toxin to inhibit binding of the iodinated toxin by 50%. At least 12 μg of cold competitor toxin was needed to achieve 50% inhibition when C2D macrophages were used. Scatchard analysis of C2D macrophage superantigen binding data indicated that a linear line best fit the data indicative of a single binding site class (Slide Write Plus, revision 4; Advanced Graphics Software, Sunnyvale, Calif.) (Fig. 2). The affinity for superantigen of the non-MHC II receptor from both bone marrow and peritoneal macrophages was lower than the affinity measured for the MHC II-positive cells (Table 1). SEA tended to bind C2D macrophages with higher affinity than did SEB, but the difference was not significant. No trend was evident when the numbers of receptors per cell for MHC II-positive and -negative macrophages were compared (Table 1). In contrast to the linear curves found for C2D macrophages, biphasic Scatchard curves indicative of two receptor sites (Fig. 3) were found for B6 peritoneal macrophages.

Actin polymerization in response to toxin. Exotoxin binding to MHC II-positive macrophages leads to activation (9). The cytoskeleton is important to early signal transduction processes. These events can be measured through changes in cellular F-actin concentration. Therefore, to determine if staphylococcal superantigens induce early signal transduction events, we measured changes in F-actin concentration. Exposing MHC II-positive macrophages to all four toxins induces actin polymerization (9). Similarly, unstimulated peritoneal macrophages from MHC II-negative C2D mice responded to the biologically active tripeptide F-Met-Leu-Phe (used as a positive control on the basis of previous work) (9), as well as SEA, ETA, and ETB (Fig. 4). A smaller increase in F-actin concentration occurred when C2D macrophages were stimulated with SEB. However, the changes were not as consistent as those seen with the other toxins, because some of the macrophages did not respond (Fig. 4D). Therefore, superantigens activated rapid intracellular responses in inflammatory C2D macrophages.

Effects of toxin on cytokine secretion. Normal macrophages release cytokines in response to staphylococcal exotoxins (13–15, 28, 35, 36). The alternative toxin binding molecule may play a role in macrophage activation. Therefore, we investigated the potential of exotoxins to activate C2D macrophages to secrete nitrite, TNF, and IL-6.

The results of these experiments were positive. Bone marrow macrophages from C2D and B6 mice secreted IL-6 when they were stimulated with staphylococcal exotoxins (Fig. 5). The amount of IL-6 secreted varied with each toxin stimulant. IL-6 secretion was especially high for SEA-stimulated C2D macrophages, while SEB induced the secretion of low concentrations of IL-6. LPS also induced the secretion of IL-6 by C2D and control B6 macrophages. However, the majority of the toxins were more potent stimulants than was LPS. Addition-

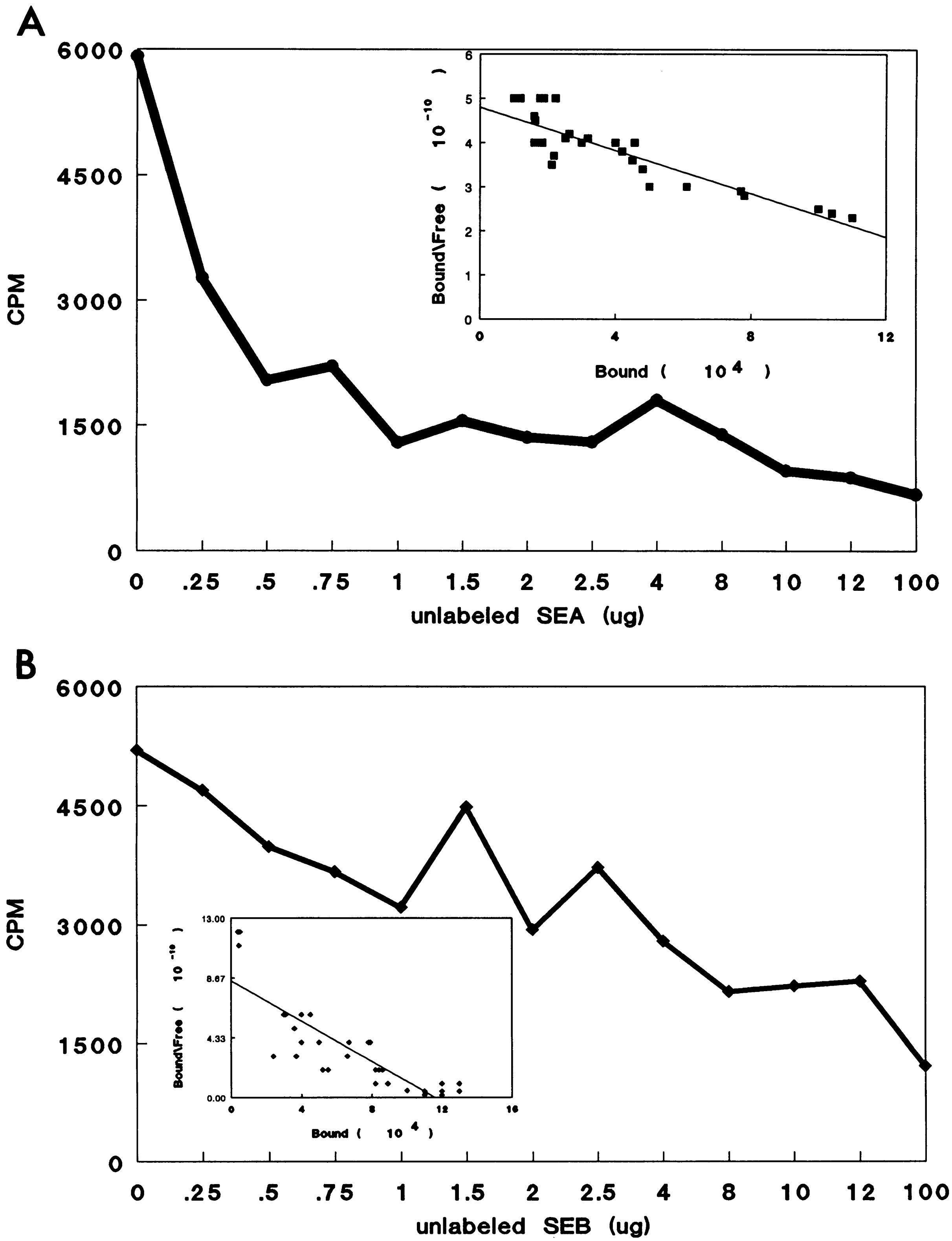

FIG. 2. Competitive inhibition of ^{125}I-SEA (A) and ^{125}I-SEB (B) binding to C2D macrophages by cold SEA or cold SEB. Scatchard plots (inserts) of these data were used to determine apparent K_ds and valences (receptors per cell). These graphs are representative of the results of at least three experiments.

TABLE 1. Binding properties of SEA and SEB for C1D, C2D (C2D-P), and C57BL/6 (B6) peritoneal and C2D (C2D-B) bone marrow macrophages[a]

Cell source	Binding of:			
	^{125}I-SEA vs SEA		^{125}I-SEB vs SEB	
	K_d	No. of receptors/cell	K_d	No. of receptors/cell
B6	1.9×10^{-7}	2.3×10^{6}	1.3×10^{-7}	2.0×10^{6}
C1D	1.4×10^{-7}	2.9×10^{6}	1.1×10^{-7}	2.6×10^{6}
C2D-P	2.0×10^{-6}	3.1×10^{6}	7.3×10^{-5}	3.9×10^{6}
C2D-B	6.6×10^{-5}	3.3×10^{6}	3.8×10^{-5}	5.1×10^{6}

[a] Values are means of results of at least three experiments in which K_d and number of receptors per cell (valence) were determined by Scatchard analysis of competitive inhibition of ^{125}I-SEA and ^{125}I-SEB binding to macrophages by cold SEA or cold SEB (^{125}I-SEA versus SEA and ^{125}I-SEB versus SEB, respectively).

ally, SEA, ETA, and ETB stimulated IL-6 secretion by peritoneal macrophages from C2D and B6 mice (Table 2). However, the level of secretion was lower than that of bone marrow macrophages.

C2D peritoneal, but not bone marrow, macrophages stimulated with SEA, ETA, and ETB also secreted TNF (Fig. 6 and Table 2). However, the TNF response was considerably lower than that seen with MHC II-positive macrophages (Table 2). C2D peritoneal macrophages also secreted nitric oxide in response to several of the exotoxins (Table 2). Again, the secretion of nitric oxide by C2D macrophages was less than that by MHC II-positive macrophages.

To rule out endotoxin effects, macrophages were stimulated with 10 μg of exotoxin per ml for 18 h in the presence of 10 μg of polymyxin B (PMB) per ml. Secretion of IL-6 and TNF by C2D macrophages in response to exotoxins did not significantly change (Table 2). In contrast, 10 μg of PMB inhibited LPS-induced TNF and IL-6 secretion by over 70% (Table 2). Therefore, cytokine secretion by toxin-treated C2D macrophages did not appear to be due to endotoxin contamination.

Immunoprecipitation. We used cell surface labeled C2D macrophages and B6 macrophages (MHC II-positive) incubated with unlabeled SEA to identify the alternative binding sites. Rabbit anti-SEA-serum precipitated bands with molecular masses of approximately 140, 97, 61, 52, 43, and 37 kDa from C2D and B6 macrophages preincubated with SEA (Fig. 7). Additionally, a very light band with a molecular mass of approximately 28 kDa was precipitated from B6 macrophages. In the absence of SEA, no cell surface molecules were precipitated (Fig. 7).

DISCUSSION

We have presented strong evidence that one or more alternative receptors exist on macrophages for staphylococcal superantigens. Our unique findings are supported by the observations of other investigators. Komisar et al. (30) reported that SEB could stimulate rat and mouse mast cells to release serotonin, but the binding site for SEB was not an MHC II molecule. Herrman et al. (24) reported that both human and mouse MHC II-negative target cells could be recognized (i.e., lysed) in a staphylococcal exotoxin-dependent fashion by cytotoxic T-lymphocyte (CTL) clones. Different staphylococcal enterotoxins were recognized preferentially by CTL on MHC II-positive and -negative targets. However, not all MHC II-negative cells could present superantigen to CTL, whereas all MHC II-positive lines tested could. Dohlsten et al. (11) reported that SEB and SEC, but not SEA and SED, could be effectively presented by MHC II-negative colon carcinoma cells. Recently, Bhardwaj et al. (4) reported that MHC II-negative, Ket/BLS1 cells showed a specific binding of ^{125}I-labeled SEA, indicating a low-affinity SEA binding site.

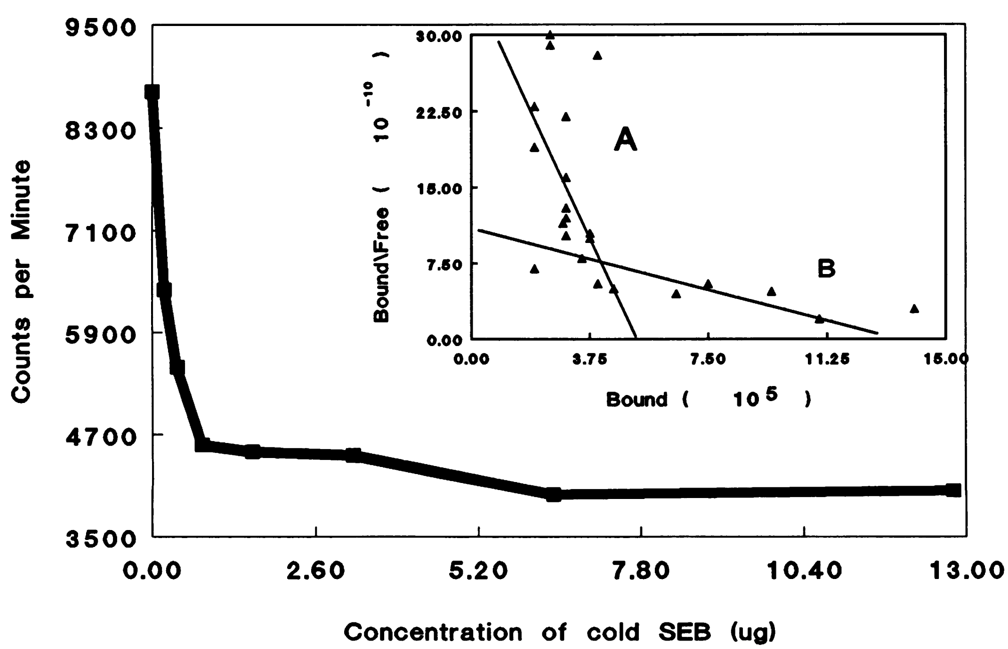

FIG. 3. Competitive inhibition of ^{125}I-SEB binding to C57BL/6 peritoneal macrophages by cold SEB. Scatchard plots (inserts) of these data were used to determine apparent K_ds and valences (receptors per cell). The line labeled A represents a K_d of 7.0×10^{-8} M and 5×10^{5} binding sites. The line labeled B represents a K_d of 3×10^{-5} M and 1.3×10^{6} binding sites.

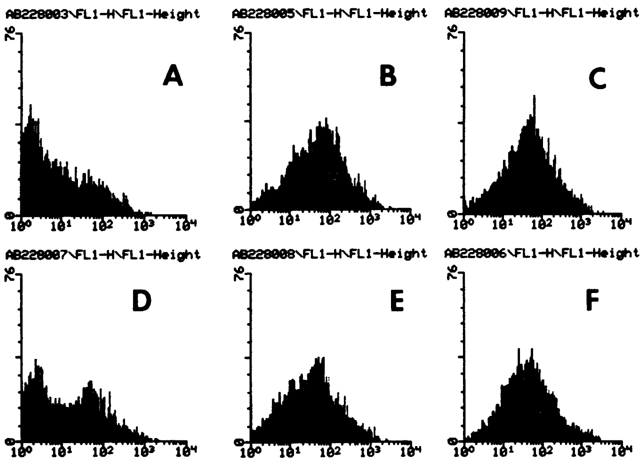

FIG. 4. Polymerized actin in C2D peritoneal macrophages was detected by using NBD-phallacidin and flow cytometry. Macrophages were incubated in PBS (A) in PBS containing F-Met-Leu-Phe (10^{-6} M) (B), or in 10 μg of SEA, SEB, ETA, or ETB per ml (C, D, E, and F, respectively). Macrophages were stimulated for 30 s and then fixed.

Viewed as a whole, the evidence indicates that molecules other than MHC II can bind staphylococcal superantigens.

Several staphylococcal exotoxins, including members from the enterotoxin and exfoliative toxin groups, bound to C2D macrophages. These data suggest that there are conserved structural features of these toxins that allow them to bind to a common receptor. It appears that the amino terminus (17, 29, 32, 37) of the enterotoxin is required for interaction with the MHC II molecule. However, no such data are available for the exfoliative toxins, nor is it known what portion of the toxin from either of these groups binds to the non-MHC II receptors. Therefore, it is not clear what these conserved toxin features are. The complex folding of the enterotoxins (42) and the suggestion that carboxyl portions of the enterotoxins (17, 20) also are necessary for MHC binding indicate that tertiary structure influences toxin-receptor interactions and does not allow for simple linear amino acid comparisons between these groups.

SEA and SEB bound to peritoneal and bone marrow-derived macrophages from C2D mice with a much lower affinity (10^{-5} M) than macrophages from either C1D or B6 control mice (10^{-8} M). This is consistent with reports of Hermann et al. (24) that found that the concentration of enterotoxin needed to lyse MHC II-negative cells was 10- to 100-fold higher than the concentration required to lyse MHC II-positive cells. Additionally, Bhardwaj et al. (4) noted that SEA binding to MHC-negative cells could be detected only at very high concentrations of toxin. In contrast, Lee et al. (31) found that TSST-1 bound to MHC II-negative porcine aortic endothelial cells with a K_d of 5×10^{-7} M. This could reflect a species difference. It has been established that human cells have a higher affinity for toxin than do mouse cells (22). Additionally, we found that toxic shock syndrome toxin 1 was, by far, the most biologically active superantigen (15); this may reflect a basic difference in toxin affinity for the non-MHC II receptor. It is also possible that different alternative receptors

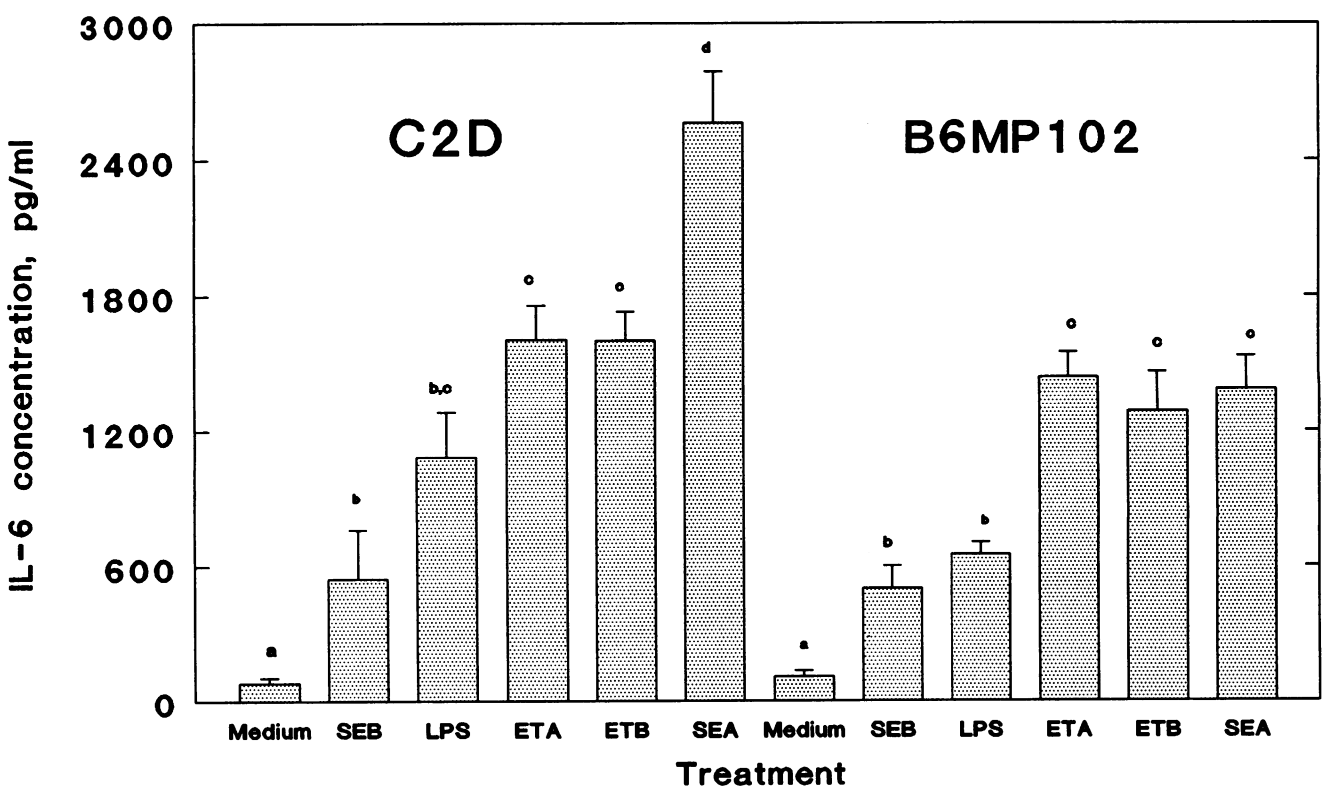

FIG. 5. Secretion of IL-6 by C2D and C57BL/6 bone marrow-derived macrophages in response to staphylococcal exotoxins (10 μg/ml) or LPS (12.5 μg/ml). Cells were stimulated for 18 h. IL-6 concentration was determined by linear regression of recombinant IL-6 standards with unknown samples. Columns with different superscripts differ (P <0.05).

TABLE 2. Summary of peritoneal macrophage activation[a]

Macrophage source and treatment	Activity in C2D cells			Activity in B6 cells		
	TNF (U/ml)	IL-6 (pg/ml)	NO_2^- (nM)	TNF (U/ml)	IL-6 (pg/ml)	NO_2^- (nM)
Medium alone	4 ± 3	296 ± 122	1 ± 1	4 ± 2	175 ± 54	8 ± 4
SEA	14 ± 5	3,100 ± 235[b]	6 ± 2	52 ± 9[b]	2,003 ± 312[b]	30 ± 12[b]
SEA + PMB	13 ± 4	3,000 ± 245[b]	ND[c]	49 ± 6[b]	1,987 ± 269[b]	ND
SEB	10 ± 2	622 ± 109[b]	2 ± 1	6 ± 5	1,222 ± 99[b]	22 ± 9[b]
SEB + PMB	9 ± 2	655 ± 100[b]	ND	6 ± 4	1,234 ± 103[b]	ND
ETA	11 ± 3	1,523 ± 264[b]	4 ± 1	24 ± 10[b]	1,600 ± 119[b]	20 ± 9[b]
ETA + PMB	10 ± 2	1,224 ± 287[b]	ND	21 ± 5[b]	1,523 ± 123[b]	ND
ETB	16 ± 1[b]	2,342 ± 387[b]	6 ± 2	23 ± 8[b]	1,700 ± 298[b]	22 ± 11[b]
ETB + PMB	14 ± 3	2,007 ± 284[b]	ND	21 ± 9[b]	1,593 ± 356[b]	ND
LPS	50 ± 5[b]	1,467 ± 236[b]	12 ± 3[b]	76 ± 4[b]	1,434 ± 216[b]	95 ± 12[b]
LPS + PMB	15 ± 7	675 ± 87[b]	ND	20 ± 3[b]	621 ± 132[b]	ND

[a] Mean activity ± standard deviation from culture supernatants of peritoneal macrophages incubated with and without exotoxin- or endotoxin-containing medium with and without polymyxin B (PMB) for 18 h.
[b] Different from medium (P <0.05).
[c] ND, not determined.

have been identified. Further characterization of the toxin receptor on C2D macrophages should resolve this issue.

The toxin binding affinity reported for normal B6 peritoneal macrophages in this study was similar to those reported for other MHC II-positive cells (7). However, if the alternative binding receptor is present on normal cells, as indicated by the biphasic nature of the Scatchard analysis, this value represents the binding of both receptors. We hypothetically divided the two receptor classes presumed to be found on normal macrophages and calculated the dissociation constants (Table 3). The high-affinity receptor probably represents MHC II. Interestingly, the low-affinity receptor has a binding constant that resembles the toxin receptor on C2D macrophages. For example, the low-affinity toxin receptor on normal macrophages had an affinity of 9×10^{-5} M for SEB, compared with an affinity of 5×10^{-5} for the SEB toxin receptor on C2D macrophages. It should be noted that the amount of toxin (measured in nanomoles) often used in receptor analysis was usually below the amount of toxin (measured in micromoles) needed to detect the alternative receptors which we have characterized in our laboratory. Therefore, it is likely that these alternative molecules are simply overshadowed by the higher-affinity MHC II molecule.

Binding of staphylococcal exotoxins to C2D macrophages leads to cellular activation in much the same way normal macrophages respond (14, 15). After toxin binding, signal transduction occurred rapidly (within 30 s) and was subsequently followed by macrophage secretory activity. Interestingly, toxin-induced IL-6 secretion in C2D macrophages equalled or exceeded that seen with normal B6 macrophages. This was not true for the TNF or nitric oxide secretory responses, suggesting that the low-affinity toxin-binding mole-

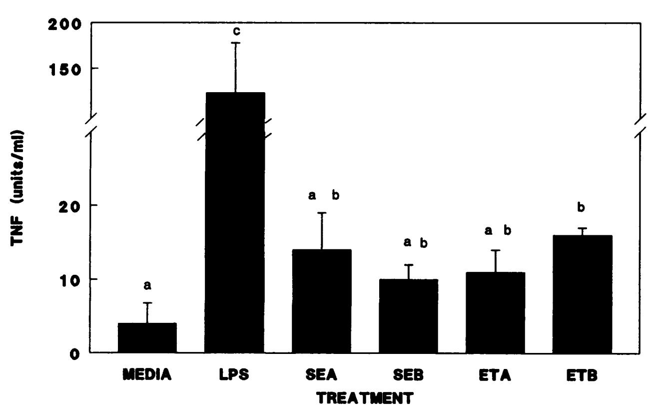

FIG. 6. Secretion of TNF by C2D peritoneal macrophages in response to staphylococcal exotoxins (10 μg/ml) or LPS (12.5 μg/ml). Cells were stimulated for 18 h. TNF concentration was determined by linear regression of recombinant murine TNF standards with unknown samples. Columns with different superscripts differ (P < 0.05).

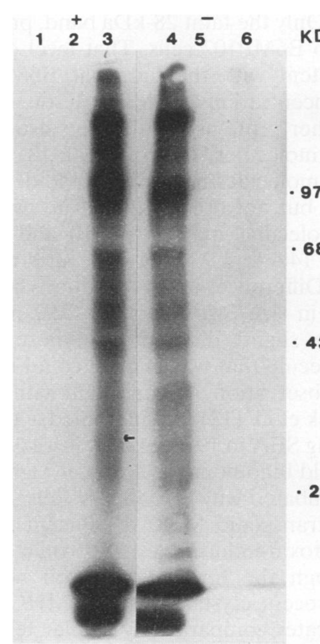

FIG. 7. Cell surface ^{125}I-labeled B6MP102 and C2D macrophages alone (lanes 1 and 6, respectively) or incubated with unlabeled SEA (lanes 3 and 4, respectively) were immunoprecipitated with rabbit anti-SEA-serum. Additionally, labeled cells were incubated with SEA and immunoprecipitated with Pansorbin (lanes 2 and 5, respectively). The arrow points to the 28-kDa band.

TABLE 3. Comparison of the toxin binding the affinities of receptors on normal and C2D macrophages[a]

Toxin	Binding affinity of receptors on:					
	Normal macrophages				C2D macrophages[b]	
	MHC II receptor		Alternative receptor			
	K_d	No. of binding sites	K_d	No. of binding sites	K_d	No. of binding sites
SEA	8×10^{-8}M	4×10^5	8×10^{-5}M	1×10^6	3×10^{-5}M	3×10^6
SEB	7×10^{-8}M	3×10^5	9×10^{-5}M	1×10^6	5×10^{-5}M	4×10^6

[a] Values were obtained by Scatchard analysis of at least three experiments.
[b] Values are averages from peritoneal and bone marrow C2D macrophages.

cules control responses different from those controlled by MHC II molecules. Our laboratory and others have found that SEB, compared with other enterotoxins, does not induce TNF very effectively (7, 13). A similar observation was made during this study. Interestingly, SEB was less effective than the other toxins in inducing IL-6 secretion by C2D macrophages, in contrast to the IL-6 secretory response induced by SEB stimulation of MHC II-positive macrophages with the H-2^k genotype (9). It appears that the IL-6 response is less dependent on the presence of MHC II than are other cytokine responses. Furthermore, the SEB-induced IL-6 response, through the alternative receptor(s), may be poorer because of lower affinity (Table 1) of SEB for the alternative binding sites. Thus, it is becoming evident that the macrophage response to staphylococcal superantigens is regulated by at least two distinct receptors, which display some polymorphism in the ways they bind toxin and in their signal complex secretory responses.

By immunoprecipitation, we identified a heterogeneous array of molecules on C2D peritoneal macrophages capable of binding SEA. These same proteins were present on MHC II-positive cells. Only the faint 28-kDa band, presumably MHC II, was unique to B6MP102 cells. That level of its expression would be consistent with the fact that B6MP102 does not express high concentrations of MHC II on its surface. The nature of the other proteins is still unresolved, but there are some candidate molecules. Cantor et al. (8) have suggested that VLA-4-like molecules may be capable of binding enterotoxins C1 and E but not other toxins. The two chains of this integrin have molecular masses of 110 and 150 kDa. It is possible that the 140- and 97-kDa molecules which we identified are VLA-4. Differences in gel conditions could explain the slight variations in size. Alternatively, C2D mice could have integrin molecules slightly different from those of normal mice. The 43-kDa molecule that was imaged could be MHC I. This hypothesis and observation are consistent with previous observations. Ezepchuk et al. (12) first identified a 43-kDa molecule capable of binding SEA in 1983. Fraser, in 1989 (16), suggested that MHC I would bind nonspecifically, if high concentrations of SEA were incubated with GM4672A cells. Stiles et al. (40) suggested that transgenic MHC I-deficient mice were less sensitive to exotoxin-induced lethality than were wild-type mice, even though the former expressed normal MHC II molecules. The recent crystallization of MHC I and II molecules (5, 6) indicates comparatively similar tertiary structures in these two molecules, especially in the nonpolymorphic regions (5, 6). Furthermore, Hansen et al. (19) have suggested that MHC I engagement cooperates with cytokine receptors and contributes to IL-2- and IL-4-induced proliferation. Therefore, exotoxin binding to MHC I would be capable of contributing to cellular (19) and physiological (36) responses. Additional work will be needed to confirm the nature of the 140-, 97-, and 43-kDa molecules as well as the 61-, 52-, and 37-kDa molecules that we have presented. Now that we have proven the existence of alternative toxin receptor(s), we are working to fully define these non-MHC II molecules.

ACKNOWLEDGMENTS

Animals used in these experiments were handled in accordance with procedures approved by the Animal Care and Use Committee at Kansas State University. The authors thank Wilma Shuman for her help with the F-actin analysis.

This work was supported by the U.S. Army Research and Development Command grant DAMD 17-89-Z-9039, NIAID grant AI-17474, NASA grants NAGW-1197 and NAGW-2328, and the Kansas Health Foundation.

REFERENCES

1. **Armstrong, J. W., S. J. Simske, A. A. Beharka, S. Balch, M. W. Luttges, and S. K. Chapes.** Class I and class II major histocompatibility (MHC) molecules play a role in bone marrow-derived macrophage development. J. Leukocyte Biol. **55:**658–661.
2. **Beezhold, D., G. Best, P. Bonventre, and M. Thompson.** 1987. Synergistic induction of interleukin-1 by endotoxin and toxic shock syndrome toxin-1 using rat macrophages. Infect. Immun. **55:**2865–2870.
3. **Bergdoll, M. S.** 1983. Enterotoxins, p. 559–598. *In* C. S. F. Eason and C. Adlam (ed.), Staphylococci and staphylococcal infections. Academic Press, Inc., New York.
4. **Bhardwaj, N., J. W. Young, A. J. Nisanian, J. Baggers, and R. M. Steinman.** 1993. Small amounts of superantigen, when presented on dendritic cells, are sufficient to initiate T cell responses. J. Exp. Med. **178:**633–637.
5. **Bjorkman, P., M. Saper, B. Samraoui, W. Bennett, J. Stominger, and D. Wiley.** 1987. Structure of the human class I histocompatibility antigen, HLA-A2. Nature (London) **329:**506–512.
6. **Brown, J., T. Jardetzky, J. Gorga, L. Stern, R. Urban, J. Strominger, and D. Wiley.** 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature (London) **364:**33–39.
7. **Buxer, S., P. Bonventre, and D. Archer.** 1981. Specific receptor binding of staphylococcal enterotoxins by murine splenic lymphocytes. Infect. Immun. **33:**827–833.
8. **Cantor, H., A. L. Crump, V. K. Raman, H. Liu, J. S. Markowitz, M. J. Grusby, and L. H. Glimcher.** 1993. Immunoregulatory effects of superantigens: interactions of staphylococcal enterotoxins with host MHC and non-MHC products. Immunol. Rev. **131:**27–42.
9. **Chapes, S. K., A. A. Beharka, M. E. Hart, M. S. Smeltzer, and J. J. Iandolo.** Differential RNA regulation by staphylococcal enterotoxins A and B in murine macrophages. J. Leukocyte Biol. **55:**523–529.
10. **Dellabona, P., J. Peccoud, C. Benoist, and D. Mathis.** 1990. Superantigens interact with MHC class II molecules outside the antigen groove. Cell **62:**1115–1121.
11. **Dohlsten, M., G. Hedlund, S. Segren, P. A. Lando, T. Herrmann, A. P. Kelly, and T. Kalland.** 1991. Human major histocompatibility

complex class II-negative colon carcinoma cells present staphylococcal superantigens to cytotoxic T lymphocytes: evidence for a novel enterotoxin receptor. Eur. J. Immunol. **21**:1229–1233.

12. **Ezepchuk, Y. V., V. P. Noskova, R. D. Aspetov, A. S. Novokhatsky, and A. N. Noscov.** 1983. Binding of staphylococcal enterotoxin A (SEA) with human splenic lymphocytes. Int. J. Biochem. **15**:285–288.
13. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor. Infect. Immun. **57**:291–294.
14. **Fast, D. J., B. J. Shannon, M. J. Herriott, M. J. Kennedy, J. A. Rummage, and R. W. Leu.** 1991. Staphylococcal exotoxins stimulate nitric oxide-dependent murine macrophage tumoricidal activity. Infect. Immun. **59**:2987–2993.
15. **Fleming, S. D., J. J. Iandolo, and S. K. Chapes.** 1991. Murine macrophage activation by staphylococcal exotoxins. Infect. Immun. **59**:4049–4055.
16. **Fraser, J. D.** 1989. High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR. Nature (London) **339**:221–223.
17. **Griggs, N., C. Pontzer, M. Jarpe, and H. Johnson.** 1992. Mapping of multiple binding domains of the superantigen staphylococcal enterotoxin A for HLA. J. Immunol. **148**:2516–2521.
18. **Grusby, M. J., S. R. Johnson, V. E. Papaioannou, and L. H. Glimcher.** 1991. Depletion of CD4$^+$ T cells in major histocompatibility complex class II-deficient mice. Science **253**:1417–1420.
19. **Hansen, N. Q., T. Tscherning, and M. H. Claeson.** 1991. T-cell activation. IV. Evidence for a functional linkage between MHC class I, interleukin-2 receptor, and interleukin-4 receptor molecules. Cytokine **3**:35–41.
20. **Hedlund, G., M. Dohsten, T. Herrmann, G. Buell, P. Lando, S. Segren, J. Schrimsher, H. Macdonald, H. Sjogren, and T. Kalland.** 1991. A recombinant C-terminal fragment of staphylococcal enterotoxin A binds to human MHC class II products but does not activate T cells. J. Immunol. **147**:4082–4085.
21. **Helle, M., L. Boeije, and L. A. Aarden.** 1988. Functional discrimination between interleukin 6 and interleukin 1. Eur. J. Immunol. **18**:1535–1540.
22. **Herman, A., G. Croteau, R. P. Sekaly, J. Kappler, and P. Marrack.** 1990. HLA-DR alleles differ in their ability to present staphylococcal enterotoxins to T cells. J. Exp. Med. **172**:709–717.
23. **Herrmann, T., R. S. Accolla, and H. R. MacDonald.** 1989. Different staphylococcal enterotoxins bind preferentially to distinct major histocompatibility complex class II isotypes. Eur. J. Immunol. **19**:2171–2174.
24. **Herrmann, T., P. Romero, S. Sartoris, F. Paiola, R. S. Accolla, J. L. Maryanski, and H. R. MacDonald.** 1991. Staphylococcal enterotoxin-dependent lysis of MHC class II negative target cells by cytolytic T lymphocytes. J. Immunol. **146**:2504–2512.
25. **Howard, T., and W. Meyer.** 1984. Chemotactic peptide modulation of actin assembly and location in neutrophils. J. Cell Biol. **98**:1265–1271.
26. **Iandolo, J.** 1989. Genetic analysis of extracellular toxins of *Staphylococcus aureus*. Annu. Rev. Microbiol. **43**:375–402.
27. **Iandolo, J.** 1990. The genetics of staphylococcal toxins and virulence factors, p. 399–426. The bacteria, vol. XI. Academic Press, Inc., New York.
28. **Ikejima, T., S. Okusawa, J. W. M. van der Meer, and C. A. Dinarello.** 1988. Induction by toxic-shock-syndrome toxin-1 of a circulating tumor necrosis factor-like substance in rabbits and of immunoreactive tumor necrosis factor and interleukin-1 from human mononuclear cells. 1988. J. Infect. Dis. **158**:1017–1025.
29. **Kappler, J., A. Herman, J. Clements, and P. Marack.** 1992. Mutations defining functional regions of the superantigen staphylococcal enterotoxin B. J. Exp. Med. **175**:387–396.
30. **Komisar, J., J. Rivera, A. Vega, and J. Tseng.** 1992. Effects of staphylococcal enterotoxin B on rodent mast cells. Infect. Immun. **60**:2969–2974.
31. **Lee, P. K., G. M. Verellotti, J. R. Deringer, and P. M. Schlievert.** 1991. Effects of staphylococcal toxic shock syndrome toxin 1 on aortic endothelial cells. J. Infect. Dis. **164**:711.
32. **Mollick, J., R. McMasters, D. Grossman, and R. Rich.** 1993. Localization of a site on bacterial superantigens that determines T cell receptor beta chain specificity. J. Exp. Med. **177**:283.
33. **Mollick, J. A., R. G. Cook, and R. R. Rich.** 1989. Class II MHC molecules are specific receptors for staphylococcus enterotoxin A. Science **244**:817–820.
34. **Nisini, R., P. Matricardi, A. Fattorossiunol, R. Biselli, and R. D'Amelio.** 1992. Presentation of superantigen by human T cell clones: a model of T-T cell interaction. Eur. J. Immunol. **22**:2033–2039.
35. **Parsonnet, J., and Z. A. Gillis.** 1988. Production of tumor necrosis factor by human monocytes in response to toxic-shock-syndrome toxin-1. 1988. J. Infect. Dis. **158**:1026–1033.
36. **Parsonnet, J., R. Hickman, D. Eardley, and G. Pier.** 1985. Induction of human interleukin-1 by toxic-shock-syndrome toxin-1. J. Infect. Dis. **151**:514–522.
37. **Pontzer, C., J. Russell, and H. Johnson.** 1989. Localization of an immune functional site on staphylococcal enterotoxin A using the synthetic peptide approach. J. Immunol. **143**:280–284.
38. **Scholl, P., A. Diez, and R. Geha.** 1989. Staphylococcal enterotoxin B and toxic shock syndrome toxin-1 bind to distinct sites on HLA-DR and HLA-DQ molecules. J. Immunol. **143**:2583–2588.
39. **Scholl, P., A. Diez, W. Mourad, J. Parsonnet, R. S. Geha, and T. Chatila.** 1989. Toxic shock syndrome toxin 1 binds to major histocompatibility complex class II molecules. Proc. Natl. Acad. Sci. USA **86**:4210–4214.
40. **Stiles, B. G., S. Bavari, T. Krakauer, and R. G. Ulrich.** 1993. Toxicity of staphylococcal enterotoxins potentiated by lipopolysaccharide: major histocompatibility complex class II molecule dependency and cytokine release. Infect. Immun. **61**:5333–5338.
41. **Stuehr, D. J., and C. F. Nathan.** 1989. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. **169**:1543–1555.
42. **Swaminathan, S., W. Fury, J. Pletcher, and M. Sax.** 1992. Crystal structure of staphylococcal enterotoxin B, a superantigen. Nature (London) **359**:801–806.
43. **Taub, D., and T. Rogers.** 1992. Direct activation of murine T cells by staphylococcal enterotoxins. Cell. Immunol. **140**:267–281.
44. **White, J., A. Herman, A. Pullen, R. Kubo, J. Kappler, and P. Marrack.** 1989. The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. Cell **56**:27–35.