INFECTION AND IMMUNITY, Nov. 1991, p. 4049–4055
0019-9567/91/114049-07$02.00/0

Vol. 59, No. 11

Murine Macrophage Activation by Staphylococcal Exotoxins†

SHERRY D. FLEMING,[1] JOHN J. IANDOLO,[2] AND STEPHEN K. CHAPES[1]*

Division of Biology[1] and Department of Pathology,[2] Kansas State University, Manhattan, Kansas 66506

Received 16 May 1991/Accepted 27 August 1991

We investigated the ability of staphylococcal enterotoxins A and B, exfoliative toxins A and B, and toxic shock syndrome toxin 1 to activate macrophages. All of the toxins tested had the potential to stimulate tumoricidal activity in peritoneal macrophages from lipopolysaccharide-responsive C3HeB/FeJ mice. In contrast, none of the toxins activated cytotoxicity in lipopolysaccharide-unresponsive macrophages from C3H/HeJ mice. We also studied toxin stimulation of monokine secretion. Staphylococcal enterotoxin A, toxic shock syndrome toxin 1, and both exfoliative toxins triggered C3HeB/FeJ macrophages to secrete tumor necrosis factor alpha, but enterotoxin B induced only marginal amounts of tumor necrosis factor. All of the toxins used stimulated interleukin-6 production by macrophages from both strains of mice. Nitric oxide is produced in response to the exfoliative toxins only by the lipopolysaccharide-responsive macrophages. These results suggest that macrophages respond differently to several staphylococcal exotoxins.

Exotoxins from *Staphylococcus aureus* cause diseases such as food poisoning, scalded skin syndrome in infants, and the multisystem disease toxic shock syndrome. The toxins are serologically distinct, single-polypeptide chains, with sizes ranging from 22 kDa to approximately 35 kDa (20).

The toxins can also induce an immune response and have been termed superantigens (37). The toxins were originally described to be mitogenic (25, 36), but recent work shows that the superantigens initiate T-cell proliferation by the T-cell receptor interacting with exotoxins which are bound to class II molecules of accessory cells (24, 26, 35). Kappler et al. (22) found that T cells which express specific V_β sequences as part of their $\alpha\beta$ T-cell receptor are stimulated specifically by staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), and toxic shock syndrome toxin 1 (TSST-1).

Macrophages are also activated by these toxins. Human monocytes stimulated with SEB and TSST-1 for 1 to 6 days secreted tumor necrosis factor (TNF) (11). Beezhold et al. (2) used 20 ng of TSST-1 per ml to stimulate rat macrophages to produce interleukin-1 (IL-1). NK cells also become cytotoxic after stimulation with SEB (1).

We studied the ability of SEA, SEB, TSST-1, exfoliative toxin A (ETA), and exfoliative toxin B (ETB) to activate LPS^n (responsive) and LPS^d (unresponsive) murine macrophages. We showed activation of contact-dependent cytotoxicity, TNF and IL-6 secretion, and nitric oxide production (as measurable by the stable end product of nitrite) (NO_2^-). We also compared the kinetics of cytokine and NO_2^- production after stimulation by different toxins.

MATERIALS AND METHODS

Mice. C3HeB/FeJ (LPS^n) and C3H/HeJ (LPS^d) mice were bred in the animal facilities in the Division of Biology at Kansas State University.

Tissue culture cells. The TNF-sensitive cell line LM929 was obtained from American Type Culture Collection (Rockville, Md.). The simian virus 40-transformed cell line F5b which was cloned from cells derived from C3H.OL embryo fibroblasts ($H\text{-}2^{01}$ K^dD^k) is killed by macrophages by a contact-dependent process and has been described previously (7, 28). LM929 and F5b were cultured three times weekly in antibiotic-free Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, Md.) supplemented with 2% fetal bovine serum, 0.3% L-glutamine (Sigma, St. Louis, Mo.), and 10% Opti-MEM 1 Reduced Serum medium (GIBCO). IL-6 was quantitated with the IL-6-dependent, murine B-cell hybridoma subclone B9 which was obtained with L. Aarden's permission from R. Nordan (National Cancer Institute, Bethesda, Md.). It was cultured in Dulbecco's modified Eagle's medium supplemented with 50 μM 2-mercaptoethanol, 5% fetal bovine serum, and 10 pg of recombinant IL-6 per ml. The hybridoma did not proliferate in response to recombinant TNF, recombinant IL-1, combinations of TNF and IL-1, or any toxin used.

Reagents. Enterotoxins A and B, purified by the procedures of Bergdoll et al. (3), were obtained from Anna Johnson-Winegar (U.S. Army Medical Research and Development Command, Ft. Detrick, Md.) or Toxin Technology (Madison, Wis.). TSST-1 was obtained from Peter Bonventre (Dept. of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, Ohio). ETA and ETB were purified in our laboratories from culture supernatants of *S. aureus* UT0003 (ETA) and UT0007 (ETB). Extracellular ETA and ETB were precipitated with saturated ammonium sulfate and dialyzed against water. ETA and ETB were then purified by two cycles of preparative isoelectric focusing. All toxin preparations used in these experiments were biologically active at 100 ng/ml as determined by spleen cell proliferation assay. The exotoxins used in this study were endotoxin free as determined by the *Limulus* amebocyte lysate assay (Sigma). Our assay was sensitive to an endotoxin concentration of 0.02 ng/ml. The presence of lipoteichoic acid was determined by Isaac Ginsburg (Hebrew University Hadassah School of Dental Medicine, Jerusalem, Isreal) using hemagglutination of lipoteichoic acid-sensitized human erythrocytes (14). The toxin preparations used in this study were lipoteichoic acid negative by this method, which is sensitive to a concentration of 0.2 μg/ml. Lipid A-associated protein containing lipopolysaccharide (LPS) was obtained from David Morrison (Dept.

* Corresponding author.
† Kansas Agricultural Experiment Station publication 90-451-J.

of Microbiology, Kansas University Medical Center, Kansas City). Recombinant murine TNF and gamma interferon (IFN-γ) were obtained from Genzyme (Cambridge, Mass.). Recombinant murine IL-6 was obtained from R & D Systems (Minneapolis, Minn.). Experiments were conducted in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and 50 μg of gentamycin sulfate per ml (DME).

Cytotoxicity assay. The macrophage cytotoxicity assay was performed as described previously (8). C3HeB/FeJ and C3H/HeJ peritoneal exudate cells were obtained 4 or 5 days after injection of 1.5 ml of sterile thioglycolate broth (Difco, Detroit, Mich.). After peritoneal lavage, the peritoneal exudate cells were washed and pipetted into flat-bottom, 96-well plates at 1×10^5 to 4×10^5 cells per well. After 1 to 2 h the medium was removed and replaced with the appropriate toxin-containing medium (with or without 0.1 U of IFN-γ per ml). Approximately 10^4 chromium-51-labelled F5b cells were added to each well, and the assay mixture was incubated for 16 to 18 h. The microtiter plates were then centrifuged, and 90-μl aliquots from each well were counted in a gamma counter. The percent specific release was calculated as follows: specific release = [experimental release − spontaneous release/(maximal release − spontaneous release)]/× 100. The maximal release and spontaneous release were determined by incubating 10^4 F5b cells in 1 N HCl (maximal) or medium (spontaneous). The spontaneous release was generally less than 40%. As positive controls, macrophages from C3HeB/FeJ mice were stimulated with *Escherichia coli* O55:B5 LPS (12.5 μg/ml) (Difco) and IFN-γ (0.1 U/ml); C3H/HeJ macrophages were stimulated with lipid A-associated protein containing LPS (10 μg/ml) and with IFN-γ (10 U/ml).

Macrophage supernatants. C3HeB/FeJ and C3H/HeJ peritoneal exudate cells were obtained by peritoneal lavage as described above. The cells were plated at a density of 10^7 cells per 60-mm-diameter tissue culture plate, allowed to adhere for 1 to 2 h, and washed with DME. The appropriate toxin in 4 ml of DME was added for 30 min. After 30 min, the plates were washed three times with 1 to 2 ml of medium and 4 ml of fresh medium with or without toxin was added. At 3 and 6 h after the initial 30-min incubation period, the supernatants were collected and 4 ml fresh DME with or without toxin was added to the culture plates.

The supernatants were clarified by centrifugation, aliquoted, and used immediately or stored at −100°C until assayed. Supernatants collected from cultures pulsed with toxin-containing medium are designated (+) toxin. The supernatants designated (−) toxin came from cultures that received only DME after the initial 30-min pulse. As a positive control, C3HeB/FeJ macrophages were pulsed with LPS (12.5 μg/ml). Unresponsive C3H/HeJ macrophages were activated with lipid A-associated protein containing LPS (10 μg/ml) and IFN-γ (10 U/ml) as described previously (9). Cytokine and nitrite quantification was based on linear regression of standard curves of recombinant TNF, recombinant IL-6, or $NaNO_2$.

TNF quantification. Triplicate samples of culture supernatants were serially diluted in DME in 96-well, flat-bottom plates. To each well, 10^4 ^{51}Cr-labelled LM929 cells were added and incubated for 16 to 18 h. After centrifugation, 90-μl aliquots were quantitated for chromium release as described.

Nitrite determination. Nitrite concentration was determined by use of the Griess reagent (1% sulfanilamide, 0.1% naphylethylene diamine dihydrochloride, and 2% H_3PO_4) as described previously (30). Briefly, triplicate 100-μl supernatant samples were placed in microtiter plates. To each well 100 μl of Griess reagent was added and incubated for 10 min at 25°C. A microtiter plate reader (Cambridge Technology, Cambridge, Mass.) was used to read the A_{550}.

IL-6 quantification. The macrophage supernatants were assayed for IL-6 content by the B9 bioassay (17). The B9 cells were washed three times in IL-6-free DME to remove residual IL-6. Two thousand B9 cells were added to serially diluted, triplicate samples of culture supernatant and allowed to incubate for 3 days at 37°C. [^{3}H]thymidine (0.5 μCi per well) was added for 8 h prior to harvesting with a PHD Cell Harvester (Cambridge Technology) and liquid scintillation counting.

TABLE 1. Cytotoxicity of F5b tumor cells by exotoxin-activated macrophages

Activator[a]	% ($\bar{x}$ ± SEM)[b] specific release at:			
	10:1[c]	20:1	30:1	40:1
SEA	13 ± 0	12 ± 0	15 ± 2	29 ± 1
SEB	9 ± 0	8 ± 1	9 ± 1	18 ± 2
ETA	11 ± 1	18 ± 1	21 ± 0	21 ± 2
ETB	22 ± 0	25 ± 1	27 ± 0	19 ± 1
LPS + IFN-γ	34 ± 2	53 ± 1	58 ± 0	44 ± 1
Medium	8 ± 3	3 ± 1	2 ± 2	6 ± 0

[a] Thioglycolate-elicited macrophages were activated with toxin (10 μg/ml) or LPS and IFN-γ as described in the Materials and Methods.
[b] From triplicate samples.
[c] Macrophage-to-target-cell ratio.

RESULTS

Toxin induction of macrophage cytotoxicity. The ability of the toxins to induce cytotoxic macrophages was measured by killing of the cell line F5b, previously characterized by our laboratory (7, 28). The cytotoxicity of C3HeB/FeJ macrophages incubated with the exotoxins was variable, ranging from 0 to 30%. Table 1 is a representative experiment which shows the levels of tumoricidal activity that can be induced by each toxin. None of the toxins stimulated cytotoxicity to the same extent as LPS and IFN-γ. Table 2 shows that the toxins alone did not always induce cytotoxicity (SEA and SEB) or induced very low levels of killing (ETA and ETB). However, when this occurred with either toxin type, cytotoxicity could be induced when used in combination with suboptimal levels of IFN-γ (Table 2). In contrast, no toxin, under any condition, was able to induce C3H/HeJ macrophages to become cytotoxic (data not shown).

Toxin induction of NO_2^-, IL-6, and TNF secretion. Nitric oxide is released by activated macrophages (4, 30). To determine whether arginine metabolism was involved in toxin-induced activation, we measured the extracellular NO_2^- released in response to the toxins. SEA, SEB, or TSST-1 did not stimulate significant quantities of NO_2^- (Table 3). However, responsive macrophages incubated continuously with ETA or ETB released significant quantities of NO_2^- during an 18-h exposure (Table 3). Nitrite was not found in measurable quantities in any other supernatants (data not shown). The LPS[d] macrophages were not stimulated to release measurable nitrite by any of the staphylococcal exotoxins (data not shown).

To determine whether TNF is secreted independently of macrophage cytolytic activity, we incubated macrophages from C3HeB/FeJ mice with SEA. Within the first 3 h TNF

TABLE 2. IFN-γ enhancement of tumoricidal activity

Expt no.	Activator[a]	% ($\bar{x}$ ± SEM)[b] specific release at:		
		10:1[c]	20:1	30:1
1	ETA	6 ± 2	15 ± 3	10 ± 1
	ETA + IFN-γ	14 ± 0	48 ± 3	40 ± 6
	ETB	20 ± 2	19 ± 1	29 ± 0
	ETB + IFN-γ	28 ± 2	33 ± 1	47 ± 2
	Medium	9 ± 2	5 ± 0	6 ± 3
	IFN-γ	5 ± 2	2 ± 2	12 ± 2
	LPS + IFN-γ	26 ± 4	37 ± 3	42 ± 5
2	SEA	9 ± 4	6 ± 1	6 ± 1
	SEA + IFN-γ	11 ± 2	18 ± 7	18 ± 2
	SEB	0 ± 1	0 ± 1	0 ± 1
	SEB + IFN-γ	29 ± 2	24 ± 2	21 ± 17
	Medium	0 ± 0	1 ± 3	6 ± 1
	IFN-γ	3 ± 5	0 ± 4	0 ± 2
	LPS + IFN-γ	19 ± 4	22 ± 3	27 ± 0

[a] Thioglycolate-elicited macrophages were activated with toxin or LPS in the presence or absence of IFN-γ as described in Materials and Methods. Reagents were used in the following concentrations: ETA, ETB, SEA, and SEB, 10 μg/ml (each); IFN-γ, 0.1 U/ml.
[b] From triplicate samples.
[c] Macrophage-to-target-cell ratio.

TABLE 4. Kinetics of TNF production by exotoxin-stimulated macrophages

Expt no.	Stimulant	Stimulation conditions[a]	U of TNF secreted[b] at:		
			3 h	6 h	18 h
1	ETA	Continuous (+)	30	4	4
		30 min (−)	10	4	4
	ETB	Continuous (+)	25	40	24
		30 min (−)	6	4	4
	LPS	Continuous (+)	>200	180	>200
	Medium		4	4	4
2	SEA	Continuous (+)	148	26	2
		30 min (−)	34	2	2
	SEB	Continuous (+)	12	2	2
		30 min (−)	2	2	2
	LPS	Continuous (+)	>200	40	20
		30 min (−)	>200	27	2
	Medium		2	2	2

[a] Macrophages were incubated in the continuous presence of toxin (+) or were pulsed for only 30 min (−) with 10 μg of SEA or SEB per ml, 1 μg of ETA or ETB per ml, or 12.5 μg of LPS per ml.
[b] TNF concentrations were determined by linear regression of standard curves with recombinant murine TNF. Peritoneal macrophages were from C3HeB/FeJ mice. Supernatants were collected and assayed for TNF at the indicated times as described in the Materials and Methods. Values represent the amount of TNF produced in one representative experiment of two to four independent trials.

appeared in the culture supernatants (Table 4). After that time, TNF secretion decreased to background levels. In contrast, responsive macrophages incubated continuously with SEB secreted only marginal amounts of TNF. There appears to be a threshold exposure time, because we found that C3HeB/FeJ macrophages pulsed with SEA or SEB for only 30 min did not secrete TNF.

TNF was secreted from C3HeB/FeJ macrophages in response to both ETA and ETB stimulation. The continuous exposure to ETA induced approximately 30 U of TNF per ml over the first 3 h (Table 4). As observed with SEA, the TNF levels dropped to background for the remainder of the 18-h experiment. In contrast, ETB induced 30 to 40 U/ml during each collection period. A 30-min pulse of either exfoliative toxin was not enough to induce significant TNF secretion (Table 4).

At a concentration of 2.5 μg/ml, TSST-1 stimulated TNF secretion when C3HeB/FeJ macrophages were pulsed for 30 min or incubated continuously with toxin (Fig. 1). When responsive macrophages were continuously incubated with suboptimal concentrations (20 ng/ml) of TSST-1, the macrophages also produced 10 U of TNF per ml. C3H/HeJ macrophages were stimulated to produce a small amount of TNF (18 U/ml) only when incubated continuously with 2 μg of TSST-1 per ml (data not shown). SEA, SEB, ETA, and ETB did not stimulate LPS^d macrophage TNF production (data not shown).

TABLE 3. Nitric oxide produced by toxin-stimulated C3HeB/FeJ macrophages

Treatment[a]	($\bar{x}$ ± SEM) μM, NO_2^-[b]
SEA	1.2 ± 1.3
SEB	1.7 ± 1.5
ETA	26.0 ± 2.6
ETB	7.9 ± 0.6
TSST-1	1.9 ± 2.8
LPS	152.0 ± 43.3
Medium	0.9 ± 0.7

[a] The stimulant-containing medium was added to adhered macrophages for 18 h. The supernatant was removed and frozen at −100°C until assayed with Griess reagent.
[b] Values were calculated from the results of two to six experiments by linear regression from a standard of known quantities of nitrite.

IL-6 was induced by all of the exotoxins tested. Either a 30-min pulse or the continuous exposure of SEA stimulated the LPS-responsive macrophages to secrete increasing amounts of IL-6 over time, with the greatest accumulation occurring during the last 12 h (Table 5). IL-6 was secreted into the culture supernatant in response to SEB, though the quantities were significantly lower and secretion appeared to peak earlier (Table 5). The exfoliative toxins induced much higher levels of IL-6 than the enterotoxins (Table 5). The continued presence of ETA and ETB induced 2 or more ng of IL-6 per ml throughout the 18 h. The kinetics of cytokine secretion induced by the exfoliative toxins also were different from those of that induced by the enterotoxins. Table 5 shows that a 30-min pulse of toxin stimulated the most IL-6 to be secreted within the first 3 h, with decreasing quantities being secreted after this time point. This is the opposite of the kinetics of a 30-min SEA pulse (Table 5).

We found that macrophages from C3H/HeJ mice could be induced to secrete only IL-6 in response to toxin stimulation. Tables 5 and 6 indicate that the continuous presence of SEA stimulated much higher concentrations of IL-6 than did SEB. However, SEB induced significantly more than background levels of IL-6. As found with the LPS^n macrophages, the exfoliative toxins also induced IL-6 secretion by LPS^d macrophages. ETA and ETB induced the secretion of 24 and 55 pg, respectively (Table 6) of IL-6 per ml, which compared favorably with the levels induced by the enterotoxins. However, the macrophages were stimulated with only 1 μg of exfoliative toxin per ml as compared with 10 μg of enterotoxin per ml.

The accumulation of IL-6 in the culture supernatant over an 18-h incubation was also measured (Table 6). Macro-

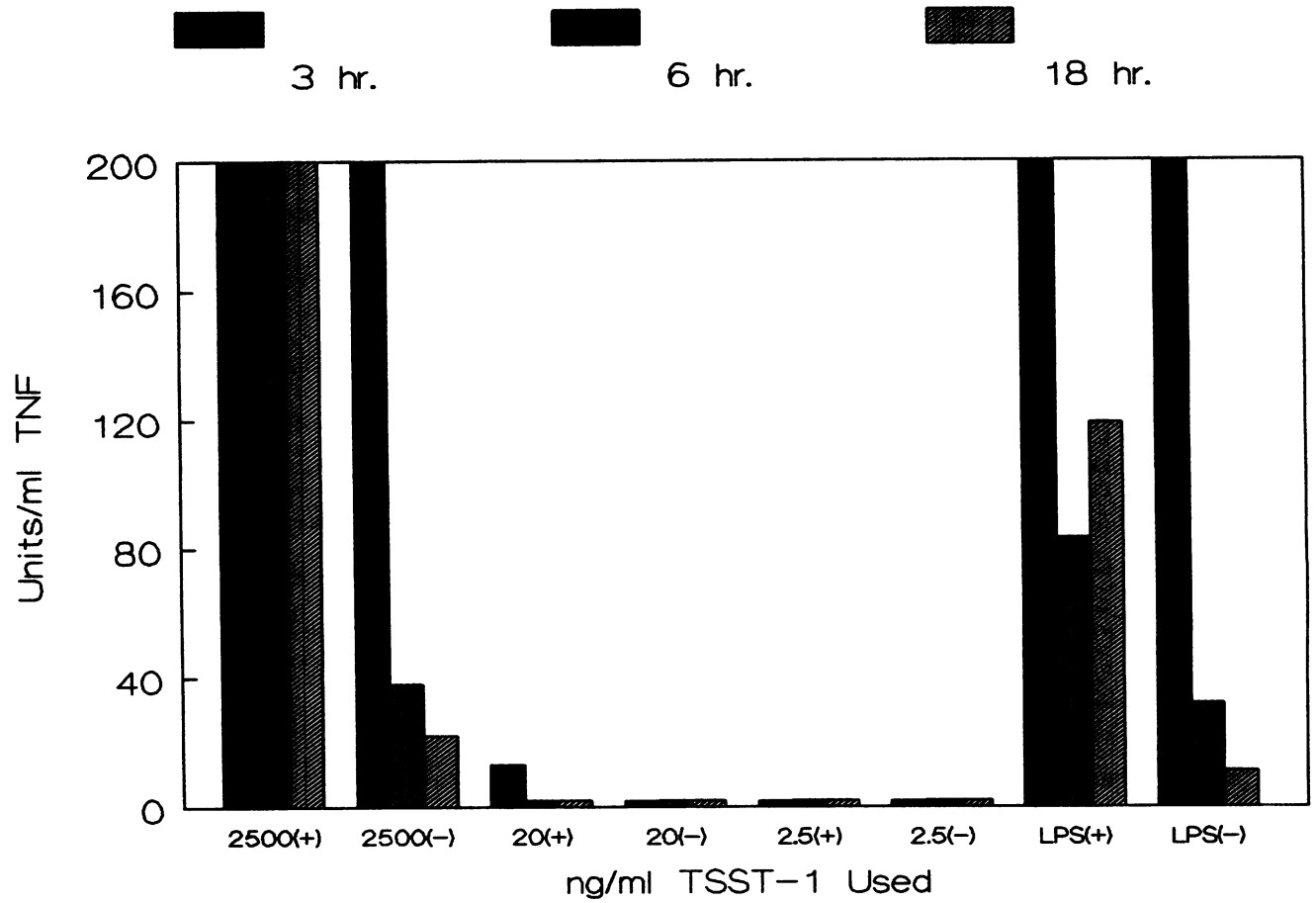

FIG. 1. Kinetics of TNF production by C3HeB/FeJ macrophages stimulated with TSST-1. C3HeB/FeJ macrophages were incubated in the presence (+) or absence (−) of TSST-1 after an initial 30-min pulse of TSST-1. Supernatants were collected and assayed for TNF at 3, 6, and 18 h as described in Materials and Methods.

TABLE 5. Kinetics of IL-6 production by exotoxin-stimulated macrophages

Stimulant	Stimulation conditions[a]	IL-6 secreted (pg/ml)[b] in: C3Heb/FeJ cells at: 3 h	6 h	18 h	C3H/HeJ cells at: 3 h	6 h	18 h
ETA	Continuous (+)	2,170	>2,400	>2,400	7	4	6
	30 min (−)	1,460	1,173	776	4	1	4
ETB	Continuous (+)	1,995	2,311	>2,400	7	9	19
	30 min (−)	1,121	558	524	4	2	6
SEA	Continuous (+)	329	718	>2,400	2	6	32
	30 min (−)	212	218	1,146	4	5	8
SEB	Continuous (+)	758	994	993	6	3	8
	30 min (−)	169	323	150	2	2	5
Medium		42	55	191	2	1	6

[a] Macrophages were incubated in the continuous presence of toxin (+) or were pulsed for only 30 min (−) with 10 μg of SEA or SEB per ml or 1 μg of ETA or ETB per ml.

[b] IL-6 concentrations were determined by linear regression of standard curves with recombinant murine IL-6. Peritoneal macrophages were from the indicated mouse line. Supernatants were collected and assayed for IL-6 at the indicated times as described in Materials and Methods. Values represent the amount of IL-6 produced in one representative experiment of two to five independent trials.

phages from C3HeB/FeJ mice exposed to a 30-min pulse or incubated continuously with ETA, ETB, or SEA secreted large quantities of IL-6. In contrast, the continued presence of SEB was necessary to stimulate greater than background levels of IL-6. The quantities of IL-6 secreted by the LPS[d] macrophages were significantly lower (Table 6). The continued presence of each toxin induced IL-6 secretion, but macrophages from C3H/HeJ mice also secreted IL-6 following a 30-min pulse of ETB or SEA. However, a pulse of SEB or ETA did not yield IL-6 in the culture supernatant (Table 6).

DISCUSSION

Staphylococcal exotoxins induce dramatic pathophysiological changes in vivo. We investigated the potential of these toxins to activate murine peritoneal macrophages, as determined by cytotoxicity and production of nitrite, TNF, and IL-6. Previous studies found that exotoxins secreted by *S. aureus* stimulated T-cell proliferation (6, 25, 26, 35, 36). Spleen cells from both C3H/HeJ and C3HeB/FeJ mice proliferated in response to 100 ng of each of our toxin preparations per ml (data not shown), confirming the biological activity of our reagents. SEA, ETA, ETB, and TSST-1 induced C3HeB/FeJ macrophages to produce substantial amounts of TNF. SEB differed from those toxins by induction of only marginal amounts of TNF. The inability of SEB to induce high levels of TNF secretion contrasts with results

TABLE 6. IL-6 secreted in response to toxin stimulation

Mouse strain	Toxin (μg/ml)	IL-6 (pg/ml)[a]	
		+Toxin	−Toxin
C3HeB/FeJ	ETA (1)	>1,600	>1,600
	ETB (1)	>1,600	>1,600
	SEA (10)	1,318	1,280
	SEB (10)	>1,600	790
	Medium		864
C3H/HeJ	ETA (1)	24	10
	ETB (1)	56	21
	SEA (10)	33	24
	SEB (10)	19	11
	Medium		14

[a] +Toxin, adhered thioglycolate-elicited macrophages were stimulated with toxin for 30 min and washed three times, 4 ml of toxin-containing medium was added, and the mixture was incubated for 18 h. The supernatant was collected, and IL-6 secretion was determined as described in Materials and Methods. −Toxin, treatment is the same as for +Toxin, except that after the wash, Dulbecco's modified Eagle's medium was added and the mixture was incubated for 18 h. The data are representative of two to five experiments, depending on the toxin.

of other recent studies. SEB and TSST-1 have recently been shown to induce human monocytes to transcribe mRNA for IL-1β and TNF (32). Fast et al. (11) also showed that TSST-1 and SEB stimulated human monocytes to produce TNF over a period of 1 to 6 days. It is possible that murine macrophages are not stimulated by SEB to the same extent as human monocytes. Murine macrophages may bind toxins differently than human macrophages because of the toxin's structure and differences in major histocompatability complex class II molecules.

Previous investigations have found that nitric oxide production and TNF secretion are concomitantly induced by the calcium ionophore A23187 (5). Macrophage activation with various toxins resulted in differential secretion of nitric oxide, TNF, and IL-6. For example, ETA and ETB were the only toxins we tested that stimulated nitrite secretion by C3HeB/FeJ macrophages. Although the amounts of nitrite produced were less than that induced by an LPS pulse, these data suggest that ETA and ETB and SEA, SEB, and TSST-1 regulate arginine metabolism differently.

We found that all toxins but SEB induced LPS-responsive macrophages to secrete greater than 15 U of TNF per ml and that only IL-6 is secreted by C3H/HeJ macrophages in response to toxin stimulation. The kinetics of nitrite, IL-6, and TNF secretion after stimulation with various toxins also differs from the response induced by the other toxins. For example, nitrite was measurable only after accumulating for 18 h in the continuous presence of exfoliative toxins, while IL-6 was measurable within 3 h and was continuously made during the 18-h incubation. In contrast, SEA induced TNF secretion within the first 3 h of stimulation, while IL-6 was not secreted in large quantities until after 6 h of exposure. Therefore, these data indicate independent regulation of nitrite, TNF, and IL-6 by these exotoxins.

The finding that some toxins can activate macrophages independently of nitrite secretion is consistent with previous studies by Green et al. (15). They found that TNF but not the macrophage activators C5 and C5a induced nitric oxide production. The production of one monokine in the absence of another or the independent regulation of monokines is consistent with a number of previous studies. For example, Riessenfeld-Orn et al. (27) stimulated human monocytes with *Streptococcus pneumoniae* to induce IL-1 but not TNF production. Lonnemann et al. (23) found that TNF and IL-1 secretion peaked at separate times when human monocytes were stimulated with LPS. Zuckerman et al. (38) also found that serum TNF peaked prior to IL-1 concentrations after in vivo stimulation. The observations that these staphylococcal exotoxins have such distinct effects on macrophages (see Table 7 for a general summary) make them interesting tools for further investigations of macrophage activation.

Tumoricidal activity induced by the toxins was variable. However, at times when no cytotoxicity was induced by the toxins, tumoricidal activity could be obtained by the same toxin preparations in conjunction with suboptimal concentrations of IFN-γ. The irregular induction of cytolytic activity may be a result of differences in the induction of IFN-α and subsequent autocrine responses. This would be consistant with multiple signals being required for macrophage cytolysis (16, 33). The finding that SEA, SEB, and TSST-1 induce cytotoxicity without NO_2^- contrasts with the results of Takema et al. (31). This difference may be attributed to the cells used in analyzing cytotoxicity. Our assays measured the cytotoxicity of F5b cells, and Takema et al. measured the killing of P815 cells. Data from our laboratory indicate that macrophage interactions with P815 cells are different from their interactions with F5b (28) and that F5b is not killed by a nitric oxide-dependent process (37a).

Macrophages from C3H/HeJ mice were not cytotoxic when stimulated with toxins. The lack of activity was not due to toxin breakdown, because the same toxin preparations stimulated spleen cells from unresponsive mice to proliferate. Furthermore, when stimulated with lipid A-associated protein containing LPS and IFN-γ, the C3H/HeJ macrophages responded as expected by becoming cytotoxic or secreting monokines.

The finding that LPS^d macrophages were not activated with the exotoxins is consistent with reports of others. C3H/HeJ macrophages do not respond to the calcium ionophore A23187 (29). They also cannot be made cytotoxic by

TABLE 7. Summary of exotoxin activation of macrophages

Macrophage source and exotoxin	Activity in C3HeB/FeJ cells (LPS responders)				Activity in C3H/HeJ (LPS nonresponders)			
	Tumoricidal activity	TNF (U/ml)[a]	IL-6 (pg/ml)[a]	NO_2^- production	Tumoricidal activity	TNF (U/ml)[a]	IL-6 (pg/ml)[a]	NO_2^- production
SEA	+	150	1,318	−	−	0	36	−
SEB	+	12	1,600	−	−	0	19	−
ETA	+	30	1,600	+	−	0	24	−
ETB	+	40	1,600	+	−	0	55	−
TSST-1	+	200	224	−	−	18	28	−

[a] In culture supernatants of macrophages incubated with toxin-containing medium for 18 h.

Propionibacterium acnes (21) or by the lipid A moiety of gram-negative bacterial LPS (10). Therefore, the genetic defect of C3H/HeJ mice that causes unresponsiveness to other stimuli extends to staphylococcal exotoxins. However, all of the toxins induced the LPS^d macrophages to secrete low levels of IL-6, and TSST-1 induced TNF secretion. The finding that unresponsive macrophages can be induced to secrete monokines without becoming cytotoxic is consistent with the results of previous studies (19). In addition, Flebbe et al. (13) found that C3H/HeJ macrophages could be stimulated by LPS isolated from rough mutants to secrete TNF and IL-1 but required an additional signal of IFN-γ to become cytotoxic. The hypothesis that distinct signals may be provided to macrophages by different toxins is not unprecedented. Smooth and rough LPS activate cells from C3H/HeJ mice differently (12, 13). The presence of protein in LPS also stimulates macrophages in a manner different from that of protein-free preparations (9, 18). We also add that though the C3H/HeJ mouse serves as an interesting model to study how these toxins work, it may not necessarily be one that reflects human disease well.

It is possible that the exotoxins stimulate different macrophage functions because they trigger autocrine responses. Vogel and Fertsch (33) found that macrophages produce IFN in response to priming signals. Macrophages require multiple signals to become fully activated (16), and each staphylococcal exotoxin may stimulate IFN differently to provide those signals. Macrophages respond differently to the various exotoxins tested (Table 7). The kinetics of TNF and nitric oxide secretion in the presence or absence of toxin also indicate that the signals transduced by each exotoxin may be distinct. Alternatively, all of the toxins may stimulate the various responses. However, structural differences may make some more potent stimulants. The conditions we chose to measure stimulation may have failed to detect the poorer activators. Additional studies will be required to elucidate whether signal transduction pathways for these toxins are indeed different.

ACKNOWLEDGMENTS

We thank Pamela Thompson and Steve Hoynowski for excellent technical assistance. We thank Isaac Ginsburg for generously testing our toxin preparations for the presence of lipoteichoic acid.

This investigation was supported by U.S. Army Medical Research and Development Command grant DAMD17-89-Z-9039, NASA grants NAGW-1197 and NAGW-2328, and NIAID grant AI-17474. S.D.F. is supported by a grant from the Wesley Foundation (Wichita, Kans.).

REFERENCES

1. **Bankhurst, A., and T. Imir.** 1989. The mechanisms involved in the activation of human natural killer cells by staphylococcal enterotoxin B. Cell. Immunol. **122**:108–121.
2. **Beezhold, D. H., G. K. Best, P. F. Bonventre, and M. Thompson.** 1987. Synergistic induction of interleukin-1 by endotoxin and toxic shock syndrome toxin-1 using rat macrophages. Infect. Immun. **55**:2865–2869.
3. **Bergdoll, M. S., H. Sugiyama, and G. M. Dack.** 1959. Staphylococcal enterotoxin. I. Purification. Arch. Biochem. Biophys. **85**:62–69.
4. **Billiar, T. R., R. D. Curran, D. J. Stuehr, M. A. West, B. G. Bentz, and R. L. Simmons.** 1989. An L-arginine-dependent mechanism mediate Kupffer cell inhibition of hepatocyte protein synthesis in vitro. J. Exp. Med. **169**:1467–1472.
5. **Buchmuller-Rouiller, Y., and J. Mauel.** 1991. Macrophage activation for intracellular killing as induced by calcium ionophore. Correlation with biologic and biochemical events. J. Immunol. **146**:217–223.
6. **Carlsson, R., H. Fischer, and H. Sjogren.** 1988. Binding of staphylococcal enterotoxin A to accessory cells is a requirement for its ability to activate human T cells. J. Immunol. **140**:2484–2488.
7. **Chapes, S., D. Duffin, and A. Paulsen.** 1988. Characterization of macrophage recognition and killing of SV40-transformed tumor cells that are "resistant" or "susceptible" to contact-mediated killing. J. Immunol. **140**:589–596.
8. **Chapes, S., and L. Gooding.** 1985. Evidence for the involvement of cytolytic macrophages in rejection of SV40-induced tumors. J. Immunol. **135**:2192–2198.
9. **Chapes, S., J. Killion, and D. Morrison.** 1988. Tumor cell killing and cytostasis by C3H/HeJ macrophages activated in vitro by lipid A-associated protein and interferon gamma. J. Leukocyte Biol. **43**:232–237.
10. **Doe, W., and P. Henson.** 1979. Macrophage stimulation by bacterial lipopolysaccharides. III. Selective unresponsiveness of C3H/HeJ macrophages to the lipid A differentiation signal. J. Immunol. **123**:2304–2310.
11. **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 production. Infect. Immun. **57**:291–294.
12. **Flebbe, L., S. W. Vukajlovich, and D. Morrison.** 1989. Immunostimulation of C3H/HeJ lymphoid cells by R-chemotype lipopolysaccharide preparations. J. Immunol. **142**:642–652.
13. **Flebbe, L. M., S. K. Chapes, and D. C. Morrison.** 1990. Activation of C3H/HeJ macrophage tumoricidal activity and cytokine release by R-chemotype lipopolysaccharide preparations: differential effects of IFN-γ. J. Immunol. **145**:1505–1511.
14. **Ginsburg, I., S. E. G. Fligiel, P. A. Ward, and J. Varani.** 1988. Lipoteichoic acid-antilipoteichoic acid complexes induce superoxide generation by human neutrophils. Inflammation **12**:525–548.
15. **Green, S. J., R. M. Crawford, J. T. Hockmeyer, M. S. Meltzer, and C. A. Nacy.** 1990. Leishmania major amastigotes initiate the L-arginine-dependent killing mechanism in IFN-γ-stimulated macrophages by induction of tumor necrosis factor-α. J. Immunol. **145**:4290–4297.
16. **Hamilton, T. A., and D. O. Adams.** 1987. Molecular mechanisms of signal transduction in macrophages. Immunol. Today **8**:151–158.
17. **Helle, M., L. Boeije, and L. A. Aarden.** 1988. Functional discrimination between interleukin 6 and interleukin 1. Eur. J. Immunol. **18**:1535–1540.
18. **Hogan, M., and S. Vogel.** 1987. Lipid A-associated proteins provide an alternate "second signal" in the activation of recombinant interferon-gamma-primed, C3H/HeJ macrophages to a fully tumoricidal state. J. Immunol. **139**:3697–3702.
19. **Hogan, M., and S. Vogel.** 1988. Production of tumor necrosis factor by rIFN-gamma-primed C3H/HeJ (Lps^d) macrophages requires the presence of lipid A-associated proteins. J. Immunol. **141**:4196–4202.
20. **Iandolo, J. J.** 1989. Genetic analysis of extracellular toxins of *Staphylococcus aureus*. Annu. Rev. Microbiol. **43**:375–402.
21. **Jackson, R., P. Lill, and J. Gangemi.** 1986. Role of pulmonary macrophages in resistance to experimental metastasis. J. Leukocyte Biol. **40**:575–587.
22. **Kappler, J., B. Kotzin, E. Gelfand, R. Bigler, A. Boylston, S. Carrel, D. Posnett, Y. Choi, and P. Marrack.** 1989. Vβ-specific stimulation of human T cells by staphylococcal toxins. Science **244**:811–813.
23. **Lonnemann, G., S. Endres, J. Van Der Meer, J. Cannon, K. Koch, and C. Dinarello.** 1989. Differences in the synthesis and kinetics of release of interleukin 1 alpha, interleukin 1 beta, and tumor necrosis factor from human mononuclear cells. Eur. J. Immunol. **19**:1531–1536.
24. **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.
25. **Morlock, B. A., L. Spero, and A. D. Johnson.** 1980. Mitogenic activity of staphylococcal exfoliative toxin. Infect. Immun. **30**:381–384.

26. **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.
27. **Riesenfeld-Orn, I., S. Wolpe, J. F. Garcia-Bustos, M. K. Hoffman, and E. Tuomanen.** 1989. Production of interleukin-1 but not tumor necrosis factor by human monocytes stimulated with pneumococcal cell surface components. Infect. Immun. **57**: 1890–1893.
28. **Rosenkrans, C. F., and S. K. Chapes.** 1991. Macrophage binding of cells resistant and sensitive to contact-dependent cytotoxicity. Cell. Immunol. **133**:187–195.
29. **Shinomiya, H., and M. Nakano.** 1987. Calcium ionophore A23187 does not stimulate lipopolysaccharide nonresponsive C3H/HeJ peritoneal macrophages to produce interleukin 1. J. Immunol. **139**:2730–2736.
30. **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.
31. **Takema, M., K. Inaba, K. Uno, K.-I. Kakihara, K. Tawara, and S. Muramatsu.** 1991. Effect of L-arginine on the retention of macrophage tumoricidal activity. J. Immunol. **146**:1928–1933.
32. **Trede, N. S., R. S. Geha, and T. Chatila.** 1991. Transcriptional activation of IL-1β and tumor necrosis factor-α genes by MHC class II ligands. J. Immunol. **146**:2310–2315.
33. **Vogel, S. N., and D. Fertsch.** 1984. Endogenous interferon production by endotoxin-responsive macrophages provides an autostimulatory differentiation signal. Infect. Immun. **45**:417–423.
34. **Vogel, S. N., and D. Fertsch.** 1987. Macrophages from endotoxin-hyporesponsive (LPS^d) C3H/HeJ mice are permissive for vesicular stomatitus virus because of reduced levels of endogenous interferon: possible mechanism for natural resistance to virus infection. J. Virol. **61**:812–818.
35. **Vroegop, S., and S. Buxser.** 1989. Cell surface molecules involved in early events in T-cell mitogenic stimulation by staphylococcal enterotoxins. Infect. Immun. **57**:1816–1824.
36. **Warren, J. R., D. L. Leatherman, and J. F. Metzger.** 1975. Evidence for cell-receptor activity in lymphocyte stimulation by staphylococcal enterotoxin. J. Immunol. **115**:49–53.
37. **White, J., A. Herman, A. M. Pullen, R. Kubo, J. W. Kappler, and P. Marrack.** 1989. The V_β-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. Cell **56**:27–35.
37a. **Woods, K., and S. K. Chapes.** Submitted for publication.
38. **Zuckerman, S., J. Shellhaas, and L. Butler.** 1989. Differential regulation of lipopolysaccharide-induced interleukin 1 and tumor necrosis factor synthesis: effects of endogenous and exogenous glucocorticoids and the role of the pituitary-adrenal axis. Eur. J. Immunol. **19**:301–305.