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Staphylococcus aureus Toxic Shock Syndrome Toxin 1 and *Streptococcus pyogenes* Erythrogenic Toxin A Modulate Inflammatory Mediator Release from Human Neutrophils

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We studied the influence of staphylococcal toxic shock syndrome toxin 1 and streptococcal erythrogenic (pyrogenic) toxin A (ETA) on intact and digitonin-permeabilized human polymorphonuclear granulocytes (PMNs). As was shown by reversed-phase high-performance liquid chromatography analysis, toxic shock syndrome toxin 1 or ETA alone, in the absence of any additional stimulus, did not induce the generation of the chemoattractant leukotriene B_4 (LTB_4) from PMNs in a wide range of concentrations. In addition, pretreatment of intact PMNs with either toxin potentiated formyl-methionyl-leucyl-phenylalanine (fMLP)- and washed *Staphylococcus aureus* cell-induced generation of LTB_4 in a time- and dose-dependent manner. This increase included LTB_4 as well as its inactive omega-oxidated compounds. Further studies revealed evidence that toxin exposure was accompanied by enhanced cellular receptor expression for fMLP as well as for LTB_4. The intrinsic GTPase activity of membrane fractions was modulated by both toxins. Short-term incubation with ETA increased the GTPase activity of PMNs up to 141%. Inhibitory effects were obtained when GTP-binding protein functions were stimulated with sodium fluoride (NaF). In addition, specific binding of Gpp(NH)p to GTP-binding protein was inhibited by both toxins during the first 10 min of incubation and was restored at later times of incubation. Our data therefore suggest that both toxins significantly affect the signal transduction pathways of human PMNs, which results in immunomodulatory functions.

Phagocytic cells such as polymorphonuclear neutrophils (PMNs) play a crucial role in host defense against bacterial infections (15). N-formylated peptides (e.g., formyl-methionyl-leucyl-phenylalanine [fMLP]), which are bacterial products with high chemotactic activities, interact with specific binding sites on the cell surface of PMNs (16). Other cellular activators, such as sodium fluoride (NaF) and the calcium ionophore A23187, interact with PMNs via defined signal transduction pathways (i.e., direct GTP-binding protein [G-protein] activation by NaF or direct elevation of intracellular calcium levels by A23187). G proteins serve as transducers for signal processing, linking extracellularly oriented receptors to membrane-bound effector systems (3, 9). By GDP-GTP exchange, they bind to distinct effector proteins, which are subsequently activated. Activation of the G protein itself is terminated by hydrolysis of bound GTP to GDP. Receptor coupling to phospholipase A_2 is one important G-protein-mediated process which leads to the release of free arachidonic acid and its subsequent conversion via a 5-lipoxygenase to leukotrienes (2, 23). Leukotrienes are potent lipid mediators which induce inflammatory and allergic reactions (5, 14, 15). For the induction of shock and sepsis, enhanced mediator release and reduced mediator release from paralyzed cells are equally important for the outcome of infection (13). Leukotriene B_4 (LTB_4) is a potent chemotactic factor for neutrophils and eosinophils, and it induces PMNs to degranulate, to generate superoxide, and to adhere to the vascular endothelium. In addition, LTB_4 exerts autocrine effects on PMNs (19). However, human PMNs are capable of metabolizing LTB_4 by specific hydroxylation at position C-20 (omega oxidation), resulting in the formation of 20-hydroxy-LTB_4 and 20-carboxy-LTB_4, which reveal diminished biological activities compared with those of LTB_4 (7, 10).

Our previous studies have indicated that microbial toxins may either induce leukotriene formation or modulate leukotriene synthesis for subsequent stimulation of the target cells (6, 12, 14). Since toxins modulate not only cellular effector functions but also G-protein-mediated signalling and cytoskeletal organization, the model of permeabilized cells was chosen for the study of these possible intracellular effects. With regard to staphylococcal toxic shock syndrome toxin 1 (TSST-1) and streptococcal erythrogenic (pyrogenic) toxin A (ETA), which belong to the family of mitogenic, shock-inducing, and superantigenic toxins (1), less is known about their abilities to induce or to modulate inflammatory mediator release from human PMNs.

The present study was designed to evaluate the effects of TSST-1 and ETA on inflammatory mediator generation and metabolization from PMNs stimulated with the bacterial chemotaxin fMLP or with washed *Staphylococcus aureus* cells.

MATERIALS AND METHODS

Materials. Ficoll 400 was obtained from Pharmacia, Uppsala, Sweden, Macrodex (6%, wt/vol) was from Knoll, Ludwigshafen, Germany, and sodium metrizoate solution (75%, wt/vol) was from Nycomed, Oslo, Norway. The cell stimuli fMLP, A23187, and NaF as well as cytochalasin B were purchased from Sigma, Munich, Germany. Acetonitrile (high-performance liquid chromatography [HPLC] grade) was provided by Baker Chemicals (Gross-Gerau, Germany),

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and methanol, dipotassium hydrogen phosphate, and phosphoric acid were from Riedel de Haën (Seelze, Germany). All other chemicals were from Merck, Darmstadt, Germany.

[^{3}H]fMLP (specific activity, 2.1 TBq/mmol), [14,15-^{3}H]LTB$_4$ (1.2 TBq/mmol), [γ-^{32}P]GTP (222 TBq/mmol), and [^{3}H]Gpp(NH)p (666 GBq/mmol) were supplied by New England Nuclear, Dreieich, Germany.

Unless stated otherwise, the buffer used throughout all experiments consisted of 0.137 M NaCl, 8 mM Na_2HPO_4, 2.7 mM KH_2PO_4, and 2.7 mM KCl (pH 7.4) (modified Dulbecco phosphate-buffered saline [PBS], referred to in this article as PBS).

Bacteria. For cellular stimulation, *S. aureus* 121C was studied. *S. aureus* strains were obtained as clinical isolates from patients hospitalized at an intensive care unit (Abteilung für Verbrennungskrankheiten und Plastische Chirurgie BG-Krankenanstalten "Bergmannsheil", Bochum, Germany). Identification of the isolates was carried out at the Institut für Medizinische Mikrobiologie, Ruhr-Universität Bochum, Germany. The strains were identified and characterized by API STAPH (Api System S.A.; Biomerieux, Marcy l'Etoile, France). Bacterial growth proceeded at 37°C in brain heart infusion broth (10 ml) which was inoculated with 100 μl of an overnight culture. Subsequently, the bacteria were centrifuged at 4,000 × *g* for 20 min, separated from the culture supernatant, and washed in PBS. Afterwards, the bacteria were reconstituted and diluted in PBS (5×10^9 bacteria per ml). The possible production of toxins by the washed bacteria could then be ruled out.

Bacterial toxins. TSST-1 and ETA were purified to homogeneity in the Microbial Toxins Unit (J. E. Alouf, Institut Pasteur) by methods described in reference 1. Both toxin preparations that had been subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue R 250 exhibited single bands corresponding to the appropriate molecular weight of each toxin. The preparations were also devoid of detectable hemolytic activity on sheep, human, and rabbit erythrocytes, indicating the absence of contamination by *S. aureus* or *Streptococcus pyogenes* hemolytic toxins.

Purification of human PMNs. Human PMNs were isolated from heparinized (15 U/ml) peripheral blood from healthy donors with a Ficoll-metrizoate gradient and subsequent dextran sedimentation as described by Böyum (4). This method leads to more than 95% pure and intact PMNs.

Cell permeabilization. Cell permeabilization was performed as described elsewhere (22). A 100-fold-concentrated solution of digitonin dissolved in water was added at a final concentration of 10 μM. The incubation proceeded at 37°C for 8 min. The PMNs were then washed twice (300 × *g* for 10 min) at 4°C in PBS, finally suspended at a concentration of 2×10^7 cells per ml of PBS, and kept on ice until they were used.

Incubation conditions. The PMNs (10^7/500 μl of PBS) were pretreated for the indicated times either with PBS as the control or with one of the toxins (TSST-1 and ETA) at 37°C; respective toxin concentrations are given in the figures and figure legends, and in every case, 10^7 PMNs in 500 μl of incubation buffer was used. Subsequently, the stimuli were added to the cell suspensions in the presence of calcium (2 mM) and magnesium (1 mM), and the incubation proceeded for an additional 20 min at 37°C. For fMLP stimulation, cytochalasin B (5 μg/ml) was added 2 min prior to the addition of fMLP.

Cell viability. In order to exclude cytotoxic reactions by the compounds used, the release of the cytoplasmic enzyme lactate dehydrogenase was measured. The release of lactate dehydrogenase after pretreatment with the indicated compounds never exceeded 4.8 ± 2.2% of the total cellular lactate dehydrogenase content (except with digitonin-permeabilized PMNs, when release was 21.0 ± 3.8%). Cytotoxicity was also assayed microscopically by the trypan blue exclusion test (7.1 ± 4.4% with pretreated intact PMNs; 29.4 ± 7.8% with digitonin-permeabilized cells).

Analysis of leukotriene generation. After the incubation period, 2 ml of methanol-acetonitrile (50:50, vol/vol) was added. The vials were overlayered with argon and frozen at −70°C for 12 h. After centrifugation at 1,900 × *g* for 10 min (Cryofuge 6-4; Heraeus Christ, Osterode, Germany), the supernatants were removed and evaporated to dryness by lyophilization (EF 4 Modulyo; Edwards-Kniese, Marburg, Germany). The precipitate was dissolved in 600 μl of methanol-water (30:70, vol/vol), and centrifugation was performed at 9,600 × *g* for 4 min for further purification. Aliquots of 200 μl were subjected to reversed-phase high-performance liquid chromatography (HPLC) analysis. The HPLC equipment consisted of a CM 4000 pump and an SM 4000 detector (Laboratory Data Control-Milton Roy, Hasselroth, Germany) and the automatic sample injector WISP 710 B (Waters, Eschborn, Germany). The reversed-phase column (4.6 by 250 mm) was packed with Nucleosil 5 C_{18} (pore size, 5 μM; Macherey-Nagel, Düren, Germany). Isocratic elution for leukotriene analysis was carried out with a solvent system consisting of water-acetonitrile-methanol (50:30:20, vol/vol/vol), including 0.04% EDTA and 0.15% K_2HPO_4. The pH was adjusted to 5.0 by the addition of phosphoric acid. The flow rate was maintained at 0.9 ml/min. All solvents were degassed before use and constantly stirred during HPLC analysis. The absorbance of the column effluent was carried out at 280 nm. Quantification and identification of leukotrienes were performed with synthetic standard solutions. LTB_4 generation was calculated by determining combined amounts of LTB_4 and the LTB_4-omega oxidation products (20-hydroxy-LTB_4 and 20-carboxy-LTB_4).

Metabolism of exogenously added LTB_4. PMNs (10^7/500 μl of PBS) were preincubated with the toxins or PBS for 10 or 30 min at 37°C. After being preincubated, LTB_4 (100 ng in 50 μl) was added to the washed-cell suspensions, and the incubation proceeded in the presence of calcium and magnesium for an additional 20 min at 37°C. Samples receiving PBS instead of LTB_4 served as controls. The reaction was stopped by the addition of 2 ml of methanol-acetonitrile (50:50, vol/vol).

Specific binding of [^{3}H]fMLP. PMNs (10^7/500 μl of PBS) were pretreated in the presence or absence of TSST-1 or ETA for the indicated times at 37°C. The binding studies were carried out with 96-well filtration plates with 5-μm-pore-size polyvinylidene fluoride membranes (Millipore, Eschborn, Germany). Each well contained 4×10^6 PMNs, 20 nM [^{3}H]fMLP, and 37.5 μg of bovine serum albumin (BSA). The nonspecific binding was assessed in the presence of 10 μM unlabelled fMLP. After 45 min of incubation at 4°C, the unbound [^{3}H]fMLP was removed by rapid filtration with a millititer vacuum holder. The filters were transferred to scintillation vials, methanol (0.5 ml) and Rotiszint 2211 (4 ml) were added, and radioactivity was measured by liquid scintillation counting (Rack beta 1209; LKB, Turku, Finland). Specific binding was expressed as total binding minus nonspecific binding. Total and nonspecific binding were calculated from triplicate determinations.

Specific binding of [^{3}H]LTB$_4$. LTB$_4$-binding studies were carried out as described above for fMLP. Each well contained 2.3 nM [^{3}H]LTB$_4$; nonspecific binding was determined in the presence of 220 nM unlabelled LTB$_4$.

Preparation of membrane fractions. PMNs (10^7/500 μl of PBS) were pretreated in the presence or absence of TSST-1 or ETA for the indicated times at 37°C. Afterwards, the cells were washed and resuspended in 0.05 M Tris buffer (pH 7.5) supplemented with sucrose (0.25 M), EDTA (1 mM), EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*,-tetraacetic acid] (1 mM), dithiothreitol (1 mM), and leupeptin (100 μg/ml). Cell disruption was carried out by sonication three times over 10 s (energy output, 40 W) (Sonifier 250 W; Branson, Danbury, Conn.). Light microscopy revealed almost complete cell breakage. The granules, nuclei, cell debris, and unbroken cells were removed by centrifugation at 10,000 × *g* for 20 min (J2-21, JA-20 rotor; Beckman, Palo Alto, Calif.). The crude membrane fractions were collected from the supernatant fraction by centrifugation at 100,000 × *g* for 60 min (Beckman centrifuge L8-70; SW60Ti rotor). The protein content was assayed according to the method of Lowry et al. (17).

GTPase activity. GTPase activity was determined by measuring the liberation of ^{32}P from [γ-^{32}P]GTP according to a modified procedure of Matsumoto et al. (18). The final assay mixture contained 10 μg of protein, 150 mM KCl, 20 mM Tris buffer (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 1.14 mM ATP, 0.5 mM App(NH)p, 0.25 mM ouabain, 0.375 μM GTP, and 0.125 μM [γ-^{32}P]GTP. The reaction proceeded for 60 min at 37°C and was terminated by the addition of 0.5 ml of a 5% charcoal mixture containing 0.1% dextran and 0.5% BSA in 20 mM phosphate buffer (pH 7.5). The tubes were then centrifuged to sediment the charcoal. The supernatants were removed, and free ^{32}P was determined by liquid scintillation.

[^{3}H]-Gpp(NH)p binding. The specific binding of [^{3}H]Gpp(NH)p to G protein was determined by a modified procedure of Matsumoto et al. (18). The membrane fraction (10 μg of protein in the reaction mixture) was incubated in 20 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1.14 mM ATP, 0.5 mM App(NH)p, 0.25 mM ouabain, and 1 μM [^{3}H]Gpp(NH)p. Nonspecific binding was defined as the amount of [^{3}H]Gpp(NH)p bound in the presence of 10 μM nonlabelled Gpp(NH)p. Incubations were terminated after 60 min at room temperature by rapid filtration through cellulose ester membranes, each with a pore size of 0.45 μm (millititer HA filtration plates; Millipore). The filters were washed four times with 20 mM Tris buffer (pH 7.5) containing 0.25 mM $MgCl_2$ and 50 μM EGTA. The dried filters were measured for radioactivity by liquid scintillation counting. The amount of specific binding of [^{3}H]Gpp(NH)p was calculated as total binding minus nonspecific binding.

Statistics. When not stated otherwise, data from at least three independent experiments with different donor cells were combined and expressed as means ± standard deviations. Student's *t* test for independent means was used to provide statistical analysis (a *P* value of >0.05 was considered not significant).

RESULTS

Effect of TSST-1 or ETA on leukotriene B$_4$ generation induced by fMLP. Under the experimental conditions described earlier, the toxins used, TSST-1 and ETA, were noncytotoxic for PMNs as assessed by the failure of lactate dehydrogenase release, indicating that the cells remained intact during the incubation and stimulation periods. Preliminary experiments showed that in the absence of any additional stimulus, the toxins did not induce the generation of inflammatory LTB$_4$ from intact and permeabilized PMNs over a wide range of concentrations. In addition, the metabolism of LTB$_4$ into its inactive omega-oxidated products was not affected by TSST-1 or after ETA exposure, as was confirmed by experiments in which the metabolism of exogenously added LTB$_4$ was studied (data not shown). In contrast, pretreatment of intact PMNs with either of the toxins potentiated the subsequent cellular activation by the bacterial chemotaxin fMLP. Compared with control incubations in the absence of the toxins, pretreatment of intact human PMNs with 500 ng of TSST-1 or ETA for 10 min at 37°C increased fMLP-induced LTB$_4$ generation up to 183 and 230%, respectively (Fig. 1, closed bars). This increase included LTB$_4$ as well as its omega-oxidated compounds.

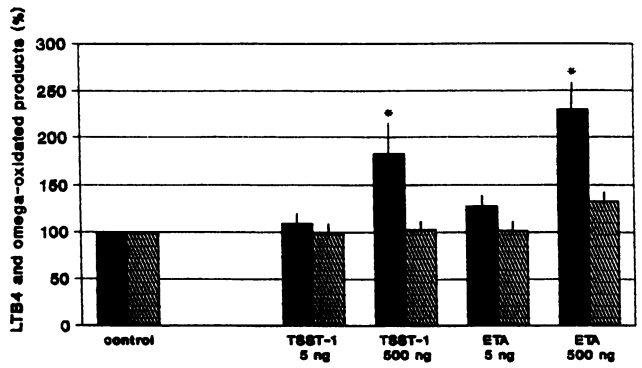

FIG. 1. Generation of LTB$_4$ from human PMNs stimulated with fMLP. Cells (10^7/500 μl of PBS) were permeabilized (▨) or nonpermeabilized (■), pretreated for 10 min with 5 or 500 ng of TSST-1 or ETA (per 500 μl of incubation buffer), and subsequently stimulated for another 20 min with fMLP (7 μM). The combined amounts of LTB$_4$ and omega-oxidated products are shown. Control incubations were performed in the absence of the toxins, and amounts of LTB$_4$ and omega-oxidated products were defined as 100%. The 100% value for fMLP-stimulated intact PMNs was 20.1 ± 8.7 ng and that for permeabilized PMNs was 38.4 ± 11.3 ng. Each value represents the mean ± standard deviation of seven independent experiments with different donor cells. *, $P < 0.01$.

In order to allow intracellular entry, subsequent experiments were carried out with permeabilized PMNs. For this purpose, cells were permeabilized by digitonin, pretreated with TSST-1 or ETA, and stimulated with fMLP as described above. As is shown in Fig. 1 (hatched bars), cell permeabilization prior to toxin exposure diminished the priming effects of both toxins, even when 500 ng of toxin was applied.

Kinetic studies with intact PMNs revealed a time optimum after 10 min of priming (i.e., enhancement of fMLP-induced leukotriene generation) for both toxins (Fig. 2A). Inhibitory effects were obtained when longer times of incubation (e.g., 120 min) were chosen. Maximal priming effects were obtained with concentrations of 500 ng of each toxin (Fig. 2B).

Effect of TSST-1 or ETA on LTB$_4$ generation induced by *S. aureus*, A23187, and NaF. In another series of experiments, we investigated the effects of both toxins on further physiological (*S. aureus*) and nonphysiological (A23187 and NaF) stimuli. In addition to the modulated cellular response to the bacterial chemotaxin fMLP, pretreatment of intact PMNs with either of the toxins significantly potentiated the gener-

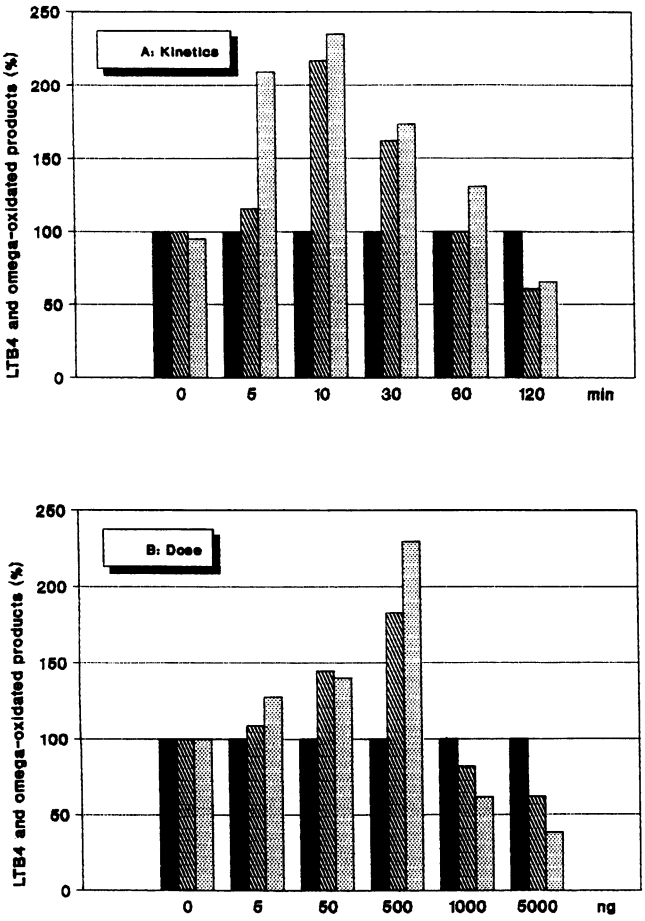

FIG. 2. Time- and dose-dependent effects for TSST-1 (▨) or ETA (□) treatment of human PMNs. Cells (10^7/500 μl of PBS) were pretreated over different times with either 500 ng of each toxin (per 500 μl of incubation buffer) (A) or different concentrations for 10 min (B). Subsequently, the PMNs were stimulated for an additional 20 min with fMLP (7 μM). The combined amounts of LTB_4 and omega-oxidated products are shown. Control incubations (■) were performed in the absence of the toxins, and amounts of LTB_4 and omega-oxidated products were defined as 100%. The 100% value for fMLP-stimulated PMNs was 20.1 ± 8.7 ng. Results represent values obtained from one typical experiment. Two experiments that showed the same pattern were carried out.

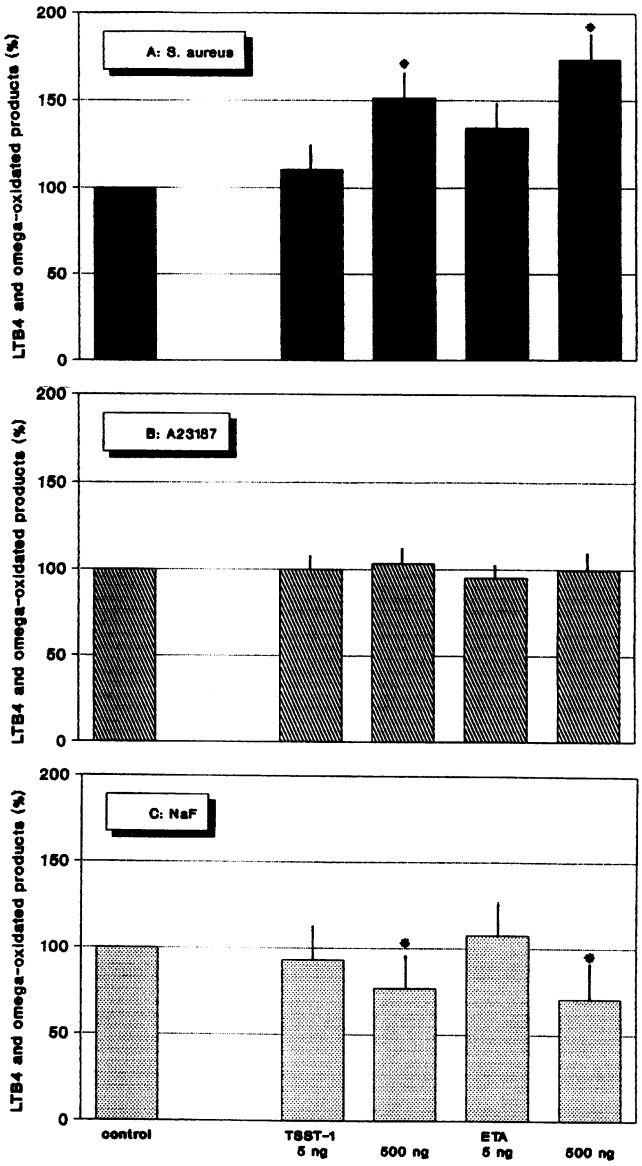

FIG. 3. Generation of LTB_4 from human PMNs stimulated with *S. aureus*, A23187, or NaF. Cells (10^7/500 μl of PBS) were pretreated for 10 min with 5 or 500 ng of TSST-1 or ETA (per 50 μl of incubation buffer) and were subsequently stimulated for an additional 20 min with washed *S. aureus* cells (2.5×10^8), A23187 (6.3 μM), or NaF (9.3 mM). The combined amounts of LTB_4 and omega-oxidated products are shown. Control incubations were performed in the absence of the toxins, and amounts of LTB_4 and omega-oxidated products were defined as 100%. The 100% value for stimulation with *S. aureus* cells was 17.5 ± 4.6 ng, that for A23187-stimulated PMNs was 259.2 ± 4.5 ng, and that for NaF-stimulated PMNs was 69.5 ± 13.0 ng. Each value represents the mean ± standard deviation of at least three independent experiments with different donor cells. *, $P < 0.01$. The horizontal axes are the same for all panels.

ation of LTB_4 induced by washed *S. aureus* bacteria (Fig. 3A). Compared with control incubations in the absence of the toxins, pretreatment of intact human PMNs with 500 ng of TSST-1 or ETA for 10 min at 37°C increased *S. aureus*-induced LTB_4 generation up to 151 and 173%, respectively. This increase included LTB_4 as well as its omega-oxidated compounds.

In contrast to these results, the stimulation of toxin-treated PMNs with the Ca ionophore A23187 was not affected (Fig. 3B); no differences in LTB_4 generation were obtained. Since the concentration of A23187 used was a maximal stimulatory dose (6.3 μM), we investigated further the abilities of the toxins to enhance LTB_4 production at suboptimal concentrations of A23187 (e.g., 3.2, 1.6, and 0.8 μM). It became apparent that the toxin effects induced by suboptimal concentrations of A23187 were comparable to the effects obtained with high concentrations of A23187; in both experimental protocols, the toxins under study did not modulate ionophore-induced LTB_4 generation (data not shown).

We then studied the direct G-protein activator NaF for cellular stimulation. Significant differences with regard to LTB_4 release were obtained when 500 ng of toxin was incubated with the cell suspensions (Fig. 3C). As was shown

TABLE 1. Effects of TSST-1 and ETA on fMLP and LTB_4 receptor expression[a]

Toxin and amt (ng/500 μl of incubation buffer)	Length of treatment (min)	% Binding[b] of:	
		[^{3}H]fMLP	[^{3}H]LTB_4
None	10	100	100
TSST-1			
5	10	135.0 ± 7	136.4 ± 13
500		148.9 ± 5	142.8 ± 13
ETA			
5	10	111.4 ± 11	140.5 ± 4
500		132.9 ± 8	147.6 ± 4
None	30	100	
TSST-1			
5	30	74.4 ± 5	
500		70.2 ± 4	
ETA			
5	30	72.8 ± 1	
500		67.1 ± 6	

[a] Cells numbered 10^7/500 μl of PBS.
[b] The binding capacities of control incubations without toxins were defined as 100%. The 100% value for [^{3}H]fMLP binding was 775 ± 82 cpm, and that for [^{3}H]LTB_4 binding was 545 ± 108 cpm. Data represent values from three independent experiments using different donor cells.

for TSST-1- or ETA-pretreated cells, NaF-induced LTB_4 generation was significantly decreased compared with that for control PMNs that had not been exposed to either toxin. Cellular pretreatment with 500 ng of TSST-1 or ETA for 10 min reduced NaF-induced LTB_4 release to 77 or 71% of controls, respectively.

Effect of TSST-1 or ETA on cellular receptor expression for fMLP and LTB_4. Human PMNs express receptor sites for fMLP and LTB_4 which are modulated during infectious processes. Since enhanced LTB_4 generation induced by fMLP may be due to an increased number of receptor sites expressed on the cell surface, experiments to analyze the effects of both toxins on receptor expression for fMLP as well as for LTB_4 were performed. Control incubations were performed in the absence of TSST-1 or ETA, and expression was defined as 100%. Our data indicate that pretreatment of PMNs with 500 ng of each toxin was accompanied by increased receptor expression for fMLP (Table 1). Pretreatment of cells with TSST-1 or ETA for 10 min increased specific [^{3}H]fMLP binding up to 149 and 133%, respectively. After 30 min of pretreatment with TSST-1 or ETA, inhibition of receptor expression was shown (70 and 67%, respectively).

Similar results were obtained for LTB_4 receptor expression after exposure to either toxin. Cellular pretreatment with 500 ng of TSST-1 or ETA for 10 min increased specific binding of [^{3}H]LTB_4 up to 143 or 148% compared with that for controls.

Effect of TSST-1 or ETA on G-protein functions. Since both toxins affected cellular stimulation by the direct G-protein activator NaF, further experiments were carried out in order to investigate G-protein functions after treatment with toxin. The activation of G proteins can be monitored as an increase in the intrinsic GTPase activity of membrane fractions by measuring the liberation of ^{32}P from GTP. In order to analyze the effect of TSST-1 or ETA on G-protein activity, PMNs were pretreated with 500 ng of each toxin for 2, 10, and 30 min (Table 2). Control incubations were carried out in the absence of the toxins, and activity was defined as 100%. As became apparent, treatment with ETA for 2 min increased the intrinsic GTPase activity of PMNs up to 141%, whereas treatment with TSST-1 showed no effects. After 10 min of incubation, GTPase activity was not modulated by either toxin. Pretreatment of the cells for 30 min led to inhibitory effects. Nearly the same pattern was obtained with fMLP-stimulated membrane fractions (data not shown).

TABLE 2. Effects of TSST-1 and ETA on G-protein functions[a]

Toxin	Length of pretreatment (min)	Value for[b]:	
		GTPase activity (%)[c]	Gpp(NH)p binding (%)[d]
None	2	100	
TSST-1	2	109.2 ± 8.9	
ETA	2	140.5 ± 2.0	
None	10	100	100
TSST-1	10	99.7 ± 3.4	78.5 ± 12.7
ETA	10	93.4 ± 4.7	83.8 ± 6.1
None	30	100	100
TSST-1	30	76.2 ± 6.4	107.9 ± 12.8
ETA	30	81.1 ± 11.2	111.5 ± 8.6

[a] Cells (10^7/500 μl of PBS) were pretreated over different times with 500 ng of TSST-1 or ETA per 500 μl of incubation buffer.
[b] Values for control incubations without toxins were defined as 100%. Data represent values from four independent experiments using different donor cells.
[c] The 100% value for GTPase activity was 103,892 ± 4,086 cpm.
[d] The 100% value for specific Gpp(NH)p binding was 1,035 ± 165 cpm.

Since it is known that the alpha subunits of G proteins exchange GDP with GTP after cellular stimulation, we analyzed the effects of both toxins on this parameter of G-protein function. The specific binding of radiolabelled Gpp(NH)p, a nonhydrolyzable GTP analog, to membrane preparations was monitored. Control stimulations were carried out in the absence of the toxins, and activity was defined as 100%. As became apparent, treatment of PMNs with 5 to 500 ng of TSST-1 or ETA reduced the specific binding of Gpp(NH)p during the first 10 min of incubation. With prolonged incubation, values for binding of Gpp(NH)p returned to control values.

DISCUSSION

A host's inflammatory response to invasion by a bacterial pathogen forms the first line of defense against infection. Any suppression of this response is likely to lead to an extended infection and to pathological damages. In this study, we provide evidence that the superantigenic toxins TSST-1 and ETA affect cellular functions of human PMNs, such as inflammatory mediator release, the expression of receptor sites for fMLP and LTB_4, and G-protein activity.

In addition to the cytolytic properties inherent to many toxins produced by pathogenic bacteria, including staphylococci and streptococci, the concept of bacterial toxin action on host organisms was recently extended to cell activation and modulation of cellular responses. In previous articles, we demonstrated that noncytolytic toxin concentrations of streptolysin O, alveolysin, and *Escherichia coli* alpha-hemolysin led to the generation of lipid mediators and oxygen radicals as well as to the modulation of different effector functions from human PMNs (6, 12, 24). The bacterial protein toxins TSST-1 and ETA possess structural and

functional homologies (1, 21). Both toxins are commonly referred to as superantigenic toxins (1). Evidence that superantigens induce distinct subsets of cytokines from T cells when they are triggered in association with major histocompatibility complex class II proteins has been previously presented (8). But it seems that their effects on human blood cells are not restricted to lymphocytes. Recently, it has been shown that TSST-1 and ETA enhance the generation of heat shock or stress proteins in human PMNs (11). Furthermore, the induction of interleukin-1 alpha and beta, interleukin-6, and tumor necrosis factor alpha from leukocytes by ETA and TSST-1 has been reported (20, 25). Since the incubation time in our experiments was usually very short (10 min), priming of the granulocytes by the respective cytokines could be ruled out. In view of the small amount of T cells and the short incubation time used, we suggest that the observed effects are due to membrane biochemical effects induced by the toxins on PMNs.

In this study, we demonstrate that TSST-1 and ETA in the absence of any additional stimulus did not directly trigger the inflammatory mediator release from human PMNs over a wide range of concentrations. This was demonstrated for permeabilized PMNs as well as for mediator release from platelets and the lymphocyte-monocyte-basophil fraction (11a). In contrast, short-term toxin exposure enhanced the inflammatory responses of intact PMNs toward bacterial stimuli (i.e., the chemotaxin fMLP and washed *S. aureus* cells, whereas long-term toxin exposure led to a diminished inflammatory response toward the bacterial stimuli without being cytolytic (Fig. 1 and 2A). This may reflect a preactivation of the cells which is followed by a cellular deactivation. Thus, increased mediator release from PMNs may also lead to unresponsiveness toward different stimuli. Such mechanisms are well known for heterologous desensitization. For the induction of shock and sepsis, enhanced mediator release and reduced mediator release from paralyzed cells are equally important for the outcome of infection and may be relevant to the pathogenicity of invading bacteria, including staphylococci and streptococci. However, the dual role of inflammatory mediators with regard to bacterial infections has been clearly demonstrated previously. Mediators support the host defense system by their chemotactic activity for phagocytes; conversely, excessive production of mediators induces a suppression of the host's immune response (5, 15).

For receptor-mediated cellular activation, the measurement of agonist-receptor binding is a prerequisite for the interpretation of data concerning the subsequent cellular response (e.g., mediator release). As we could demonstrate here, TSST-1- and ETA-induced increases in fMLP-stimulated LTB_4 generation roughly paralleled an increased expression of membrane receptors for fMLP and LTB_4. Thus, at early intervals the toxins may prime PMNs by up-regulating signal recognition molecules (Table 1). At later intervals, both toxins exerted inhibitory effects, which could imply an unresponsiveness to chemotaxins. Additional evidence that newly generated LTB_4 has the ability to positively affect leukotriene synthesis by affecting the phospholipase A_2-reesterification component of the leukotriene biosynthesis pathway in PMNs has been presented (19). Such autocrine effects may therefore represent an important amplification mechanism of leukotriene synthesis, thereby contributing to the evolution of the inflammatory response.

Using two different nonphysiologic cell stimuli (i.e., A23187 and NaF) with a well-defined biochemical activation mode, we wanted to assess which component of the signal transduction pathway was most likely affected by the toxins. When the Ca ionophore A23187, which is known to elevate the intracellular calcium level directly, was used for cellular stimulation, the inflammatory response was not affected by either toxin (Fig. 3B). In contrast, when NaF, which directly activates signal-transducing G proteins, was used as the stimulus, leukotriene formation was significantly impaired (Fig. 3C). Our results therefore imply that the toxins may interact with G-protein functions.

G proteins are a family of signal-coupling proteins that transduce signals from membrane-bound receptors to defined effector functions. In order to confirm the susceptibility of G proteins from PMNs to both bacterial toxins, we investigated two important G-protein functions: GTPase activity and Gpp(NH)p-binding capacity. Our results show that both G-protein parameters were modulated by the toxins (Table 2). Interestingly, our results indicate differences between the modulatory characters of TSST-1 and ETA. Whereas short-term incubations with ETA increased the intrinsic GTPase activity of the PMN membrane fraction, TSST-1 priming was largely without effect. Inhibitory effects on the GTPase activity of PMNs were obtained after long-term toxin exposure, suggesting a switching off of G-protein activity which was paralleled by impaired receptor expression for fMLP and LTB_4. Binding of the guanine nucleotide analog Gpp(NH)p to G proteins was already suppressed in PMNs that had been treated with the toxins for 10 min, suggesting that the G proteins remained in the active GTP-bound conformation.

Since subsequent NaF-stimulated leukotriene formation was significantly impaired, one may also consider that both toxins directly interact with or bind to G proteins, resulting in a competition for binding sites with NaF. Further studies will analyze the effects of the toxins on heterotrimeric as well as low-molecular-weight G proteins.

Another remarkable point for discussion is the extremely fast cellular dysregulation apparent after exposure to TSST-1 or ETA (Fig. 2A). This is in contrast to results with most bacterial toxins, e.g., the ADP-ribosylating toxins cholera toxin and pertussis toxin, which have to penetrate the cell membrane in order to express their enzymatic reactivities (3, 12). In fact, we could demonstrate that cells permeabilized by digitonin were no longer susceptible to TSST-1 or ETA (Fig. 1), suggesting that the effects were dependent on cellular integrity. One may therefore speculate that the effects of the toxins may be due to binding to not-yet-defined receptor structures on the cellular surface of PMNs which then induce the modulation of mediator release. Evidence that different staphylococcal superantigens bind to distinct major histocompatibility complex class II epitopes on monocytes has been previously presented (25). Target structures for TSST-1 or ETA on the surface of PMNs, which may be responsible for cellular modulation, have not been identified.

In summary, our data suggest that both toxins, TSST-1 and ETA, induce significant effects on the G protein-mediated signal transduction pathway of human PMNs, which result in immunomodulatory functions. Thus, the up-regulated inflammatory response may contribute to some characteristic disease syndromes, e.g., toxic shock, fever, and erythematous skin flush, in addition to the up-regulation of defined subsets of cytokines.

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