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Stimulation of Monokine Production by Lipoteichoic Acids

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Lipoteichoic acids (LTAs) isolated from bacterial species, including *Staphylococcus aureus*, *Streptococcus pyogenes* A, *Enterococcus faecalis*, *Streptococcus pneumoniae*, and *Listeria monocytogenes*, were tested for their ability to stimulate the production of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha in cultured human monocytes. LTAs from *S. aureus* and *S. pneumoniae* failed to induce monokine production when applied in the concentration range of 0.05 to 5.0 μg/ml. However, LTAs from several enterococcal species (0.5 to 5 μg/ml) induced the release of all three monokines at levels similar to those observed after lipopolysaccharide stimulation. The kinetics of IL-1β and tumor necrosis factor alpha release elicited by LTAs closely resembled those observed following lipopolysaccharide application. Cytokine production occurred in the presence of both fetal calf serum and autologous human serum. Hence, it was not dependent on complement activation and could not be suppressed by naturally occurring human antibodies. Deacylation caused the total loss of monocyte stimulatory capacity. Deacylated LTAs were unable to prevent monocyte activation by intact LTAs, so primary binding of these molecules probably does not involve a simple interaction of a membrane receptor with the hydrophilic portion of the molecule. The results identify some species of LTAs as inducers of monokine production in human monocytes.

Monocyte-derived cytokines serve pivotal functions in regulating a broad spectrum of cellular activities (8, 16, 17). Overproduction of interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) is implicated in the pathogenesis of septic shock (9, 17, 24), and identification of agents that stimulate their synthesis has therefore become a subject of widespread interest. The potent stimulatory properties of lipopolysaccharides (LPSs) from gram-negative organisms have been studied in great detail. However, we are not aware of any systematic study of the possible stimulatory action of cell surface components derived from gram-positive organisms. Incubation of monocytes with whole staphylococci stimulates the release of IL-1β (33), and a stimulatory effect of isolated staphylococcal lipoteichoic acids (LTAs) has also been reported (32). Pneumococcal LTA purportedly stimulates IL-1β production but not TNF-α release by human monocytes (37). In the present investigation, we conducted a systematic investigation of the release of IL-1β, IL-6, and TNF-α by human monocytes in response to a variety of LTAs, including those isolated from major, medically important pathogens. We observed a surprising divergence in the capacities of these molecules to provoke cytokine release. Certain LTAs provoke marked stimulation of all three cytokines, so these molecules may represent significant virulence factors of the respective pathogens, including several enterococcal strains.

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MATERIALS AND METHODS

Preparation of monocytes. Blood was drawn with heparin (final concentration, 50 U/ml) from healthy donors, and 5-ml aliquots were placed in 4 ml of Ficoll (Pharmacia, Uppsala, Sweden) in sterile plastic tubes. After 20 min of centrifugation at 2,400 rpm and 15°C (model GPKR; Beckman, Dreieich, Germany), cells from the intermediate phase were recovered, washed twice with RPMI 1640 medium with L-glutamine (GIBCO Laboratories, Eggenstein, Germany) at 1,800 rpm for 5 min at 15°C, and resuspended in medium. After another centrifugation at 800 rpm for 10 min at 15°C, cells were resuspended at 3×10^6/ml of medium. Aliquots (200 μl) were placed in 96-well microtiter culture plates (Nunc, Wiesbaden, Germany) and incubated for 1 h at 37°C with 5% CO_2. Thereafter, cells were washed twice with RPMI 1640 medium. Estimates of cell numbers in the culture wells were obtained by counting with a calibrated ocular microscope and by quantitation of cellular ATP as described previously (10). Generally, cell numbers ranged from 2×10^4 to 4×10^4 cells per well.

Treatment of monocytes with stimuli. Adherent monocytes were treated with LTA (diluted in RPMI 1640 medium plus 2.5% fetal calf serum or 10% autologous serum) at a concentration of 0.05 to 5.0 μg/ml for various times. Polymyxin B was added at a final concentration of 2.5 μg/ml. Poly-L-arginine and poly-L-lysine were obtained from Sigma, Munich, Germany. *Staphylococcus aureus* alpha-toxin was prepared in our laboratory (10). LPS (from *Escherichia coli* O55:B5) was purchased from Sigma.

LTAs. Some of the LTAs (11, 30) and the succinylated lipomannan of *Micrococcus luteus* (20) were from previous work and were characterized in the references given. Enterococci were grown, harvested, mechanically disintegrated, and extracted with hot phenol water as described previously (21, 23). Their LTAs were purified from crude extracts by hydrophobic interaction chromatography on octyl-Sepharose (21, 23), and fractions containing species with two and four fatty acids were collected separately (23). For these LTAs, lipid structures, average lengths, and substitutions of hydrophilic chains were determined by established procedures (19, 23). Growth of *Streptococcus pneumoniae* and isolation and structural characterization of pneumococcal LTA will be described elsewhere (7a). The LTAs purified by hydrophobic interaction chromatography

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(20, 21) did not contain detectable amounts of lipid, nucleic acid, or protein contaminants. On a weight basis, the detection limit for these contaminants was less than 0.1% (20). Pneumococcal LTA exhibited high Forssman antigenicity when tested in a passive hemolysis inhibition assay (12, 25, 28).

LTAs were deacylated by treatment with 0.2 NaOH at 37°C for 1 h. Thereafter, samples were neutralized with acetic acid and used directly in monocyte stimulation assays. Alanine ester was removed from LTA by treatment with 0.1 M glycine amide buffer (pH 8.5) at 37°C for 24 h (22), and the deacylated LTA was converted into sodium salt form and purified by hydrophobic interaction chromatography with a centrifuge filtration device as previously described (23).

Measurements of cytokines. IL-1β, IL-6, and TNF-α were assayed in cell supernatants by enzyme-linked immunosorbent assays obtained from Medgenix Diagnostic, Ratingen, Germany. Additionally, IL-1β determinations were also performed by use of an immunoradiometric assay (Medgenix). Both assays yielded equivalent results. Concentrations were expressed in nanograms per milliliter and were calculated from calibration curves obtained with the standards supplied with each kit.

TABLE 1. Capacity of LTAs to stimulate monokine production

Tested strain	Stimulatory activity[a]
Bacillus megaterium ATCC 14581	+
Bacillus subtilis W23	+
Enterococcus avium DSM 20679[b]	++
Enterococcus casseliflavus DSM 20680	++
Enterococcus durans DSM 20633[b]	+
Enterococcus faecalis Kiel 27738[b]	++
Enterococcus faecalis DSM 20478[b]	+
Enterococcus faecium DSM 2918[b]	++
Enterococcus hirae ATCC 9790[b]	++
Enterococcus hirae NCTB 8191	+
Enterococcus malodoratus DSM 20681[b]	++
Lactococcus lactis NCDO 712	+
Leuconostoc mesenteroides DSM 20343	−
Listeria monoytogenes NCTC 7973	++
Micrococcus luteus ATCC 4698	++
Staphylococcus aureus DSM 20233	−
Streptococcus mutans NCTC 10449	−
Streptococcus pneumoniae R6	−
Streptococcus pyogenes II/D 698	+
Streptococcus sangius DSM 20567	++

[a] −, negative; +, positive; ++, strongly positive.
[b] LTAs with two and four fatty acids.

RESULTS

LTAs derived from some bacterial species stimulate monokine production. Incubation of freshly isolated human monocytes with purified LTAs in the presence of either fetal calf serum or autologous serum and polymyxin B for 6 to 20 h resulted in different responses, depending on the source of the material. Cytokine responses were dose dependent and maximal at LTA concentrations of 0.5 to 5 μg/ml. No LTA provoked a cytokine response when used at a concentration of ≤50 ng/ml. Discrepancies in the production of the individual monokines tested were never noted; i.e., the production of one cytokine was always accompanied by the production of the others. This finding contrasted with that from another laboratory, in which selective stimulation by pneumococcal LTA of IL-1β production in the absence of a TNF-α response was noted (37). The following patterns were observed (Table 1). One group of LTAs failed to evoke significant cytokine production, even when used at the highest dose (50 μg/ml). This group included LTAs from *S. aureus*, *Streptococcus mutans*, and *S. pneumoniae*. Strong positive responses were observed with LTAs from most enterococcal strains, whereas intermediate responses were obtained with materials obtained from a few enterococcal strains and from *Bacillus subtilis*, *Lactococcus lactis*, and *Streptococcus pyogenes* A. "Positive" implied that the elicited monokine concentrations were ≧50% those observed after stimulation with 1 μg of LPS per ml. Typically, the concentration of each monokine was in the range of 4 to 8 ng/ml in 100 μl of supernatant (obtained from 2×10^4 to 4×10^4 cells in one well). Values below 10% the maximal reference LPS concentration were considered negative, while values between 10 and 30% the LPS-induced concentrations were categorized as intermediate. Generally, stimulatory LTAs evoked the production of TNF-α and IL-6 in concentrations similar to those elicited by LPS, whereas IL-1β responses tended to be lower (30 to 50% maximal responses to LPS).

Kinetics of cytokine release. The kinetics of LTA-induced release of IL-1β and TNF-α were similar to those observed after LPS stimulation. TNF-α release commenced after 1 to 2 h of incubation and approached the maximum after 3 to 4 h. IL-1β release was more protracted, commencing after an incubation period of approximately 3 h and approaching the maximum after 6 to 8 h (Fig. 1). Monocytes cultured for 12 to 24 h lost their capacity to respond to LTA stimulation, just as they became unresponsive to LPS (data not shown).

Deacylated LTAs are devoid of stimulatory activity. Deacylated derivatives of LTAs from *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae*, and *Listeria monocytogenes* and the deacylated lipomannan of *M. luteus* were tested and uniformly found to be utterly devoid of stimulatory activity. To discern whether these molecules might still interact with and block specific binding sites, we performed experiments in which cells were preincubated with deacylated LTAs (5 μg/ml, 2 h) and then intact LTAs (5 to 0.5 μg/ml) were added to the cultures. In no instance did we ever observe any inhibitory effect of the deacylated LTAs. Since deacylation was done by very mild alkaline treatment (0.2 M NaOH, 37°C, 1 h) and the deacylated products were tested without any separating step, the absence of monokine production strongly argued against contaminants having effected the positive reactions with the intact LTAs.

In another set of experiments, cells were preincubated with weakly stimulatory LTAs from *E. faecalis* DSM for 2 h (5 μg/ml) and then strongly stimulatory LTAs from *E. faecalis* Kiel (0.25 to 2.5 μg/ml) were added. These experiments also yielded no indication of the inhibition of cytokine production by preincubation of cells with weakly stimulatory LTAs.

Polycations block the stimulatory actions of LTAs. The above-described findings demonstrated an essential role for fatty acids in mediating the stimulatory effects of LTAs. To assess whether the negatively charged, polar moiety was also important, we added LTAs to monocytes in the presence of poly-L-arginine or poly-L-lysine. These agents were found to completely suppress the stimulatory actions of

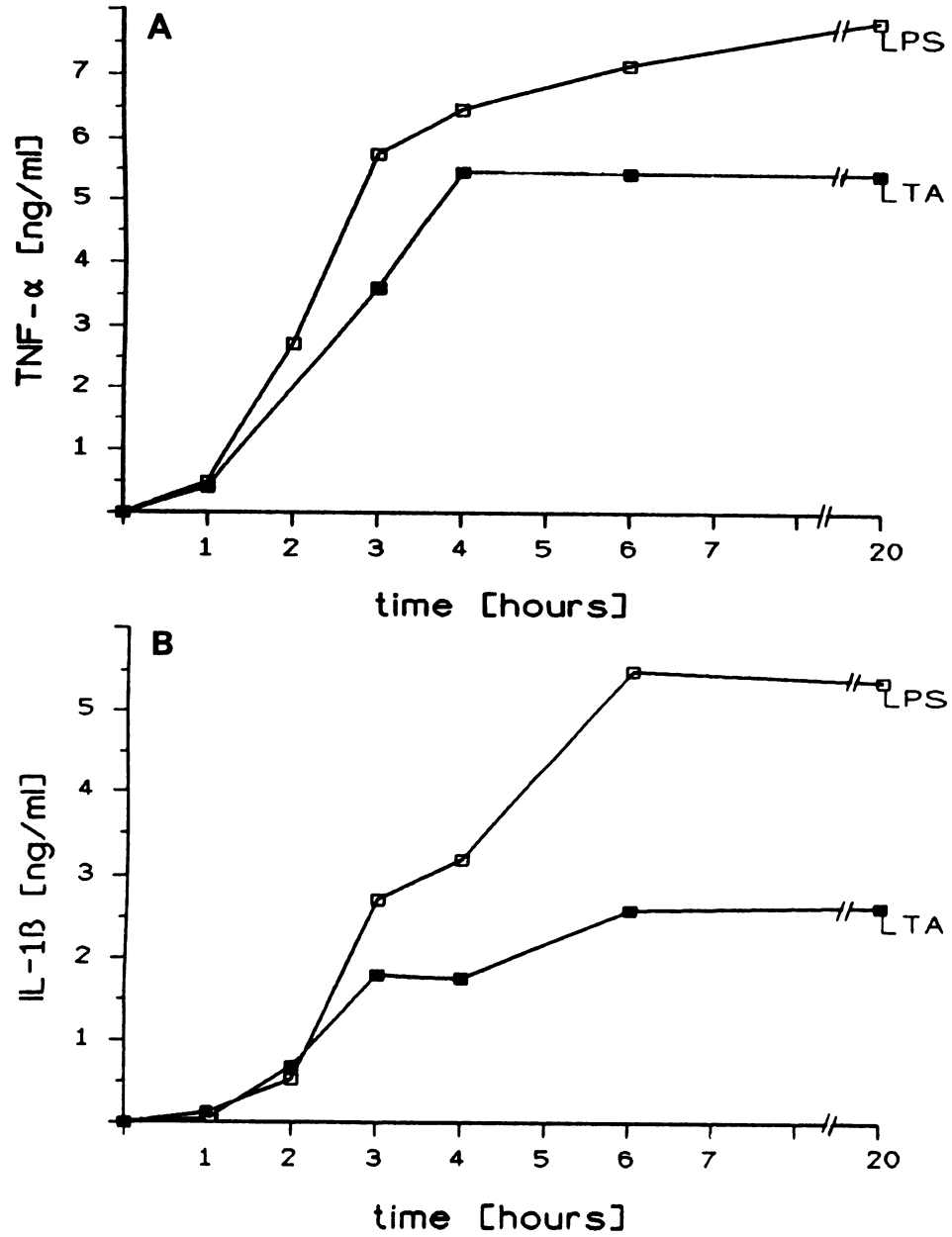

FIG. 1. Kinetics of release of TNF-α (A) and IL-1β (B) from human monocytes induced by LPS (1 μg/ml) and LTA (5 μg/ml). The LTA used in this experiment was from *E. faecium*.

LTAs (Fig. 2). Significantly, however, neither polycation used at 10 μg/ml blocked the effects of LPS (used at 1 to 100 ng/ml).

In another set of experiments, cells were preincubated with LTAs for various periods (1 to 120 min). Supernatants were harvested for cytokine determinations and replaced with LTA-free medium with or without poly-L-arginine, and cytokine determinations were subsequently performed with supernatants after 6 h of incubation. It was found that short contact times, i.e., 5 to 30 min, sufficed to trigger cytokine production in every case, with levels attained in supernatants Fig. 3, (open columns) reaching 40 to 60% those in controls (6 h of uninterrupted incubation with LTAs) (Fig. 3). On the basis of these experiments, we concluded that LTAs bind initially in a fairly rapid manner to cells. Furthermore, because poly-L-arginine was unable to inhibit cytokine production when used after the binding step, the inhibitory action of the polycations was due to their inhibition of cell binding, probably via an electrostatic interaction with negatively charged groups of LTAs.

Lack of common structural motifs among stimulatory LTAs. Analysis of the chemical structures of the stimulatory versus the nonstimulatory LTAs failed to reveal any common structural motifs that might explain their divergent actions. All stimulatory LTAs carry glycosyl substituents on their poly(glycerophosphate) chains. The enterococcal LTAs and the LTA of *Streptococcus sanguis* contain α-D-glycopyranosyl residues and α-D-gluco-oligosaccharides, the

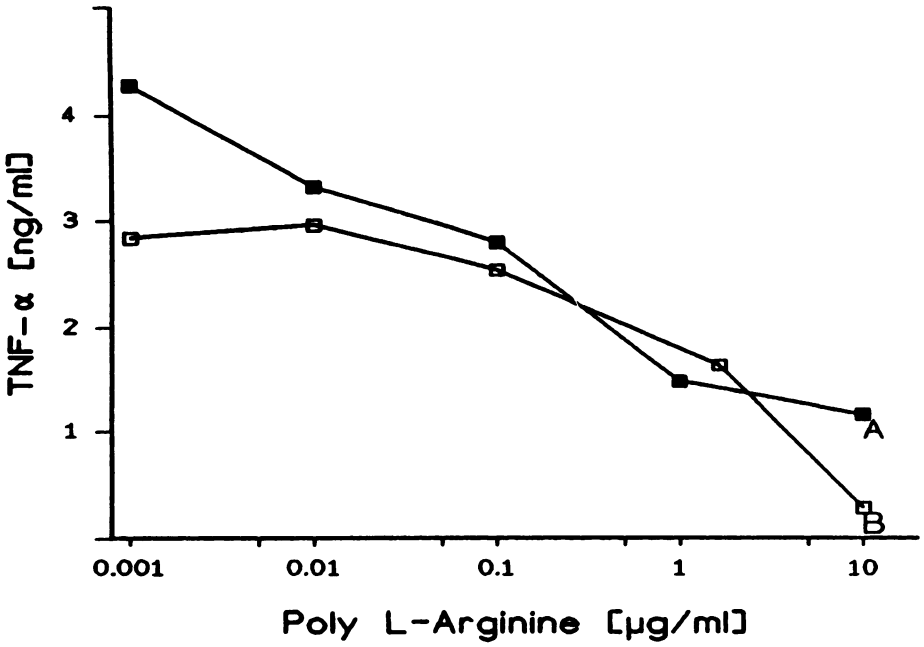

FIG. 2. Inhibition by polycations of the stimulatory actions of LTAs on monocytes. Cells were incubated with 0.5 μg of LTA from *E. faecium* per ml in the presence of poly-L-arginine at the indicated concentrations, and TNF-α was assayed in the cell supernatants after 6 h. The curves represent the data obtained with two donors (A and B). Control values obtained in the absence of the polycation were similar to those obtained with 0.001 μg of poly-L-arginine per ml. Essentially the same results were obtained with poly-L-lysine.

LTA of *L. monocytogenes* contains α-D-galactopyranosyl residues, and the LTA of *B. subtilis* contains α-*N*-acetyl-D-glucosaminyl residues. The same substituents occur, although to a lesser extent, on the nonstimulatory LTAs of *Leuconostoc mesenteroides* and *S. aureus*. When one further compares the reactivities with monocytes of various enterococcal LTAs and their fine structures, summarized in Table 2, there is no obvious relationship between reactivity

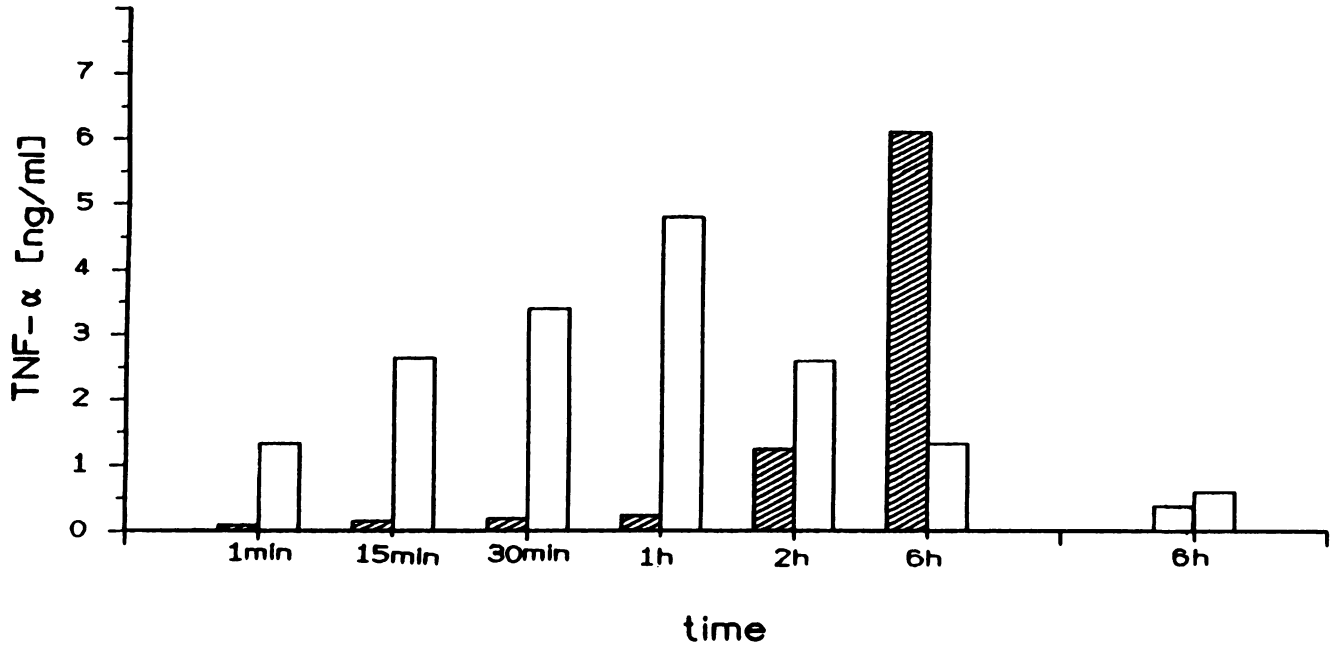

FIG. 3. Triggering by short periods of contact with LTAs of monokine production in monocytes. LTA from *E. hirae* (0.5 μg/ml) was applied to monocytes for the indicated time periods, and the cell supernatants were assayed for TNF-α (shaded columns). The cells received LTA-free medium, and TNF-α was determined in the supernatants after 6 h of incubation at 37°C (open columns). The polycation would have blocked any action of residual, free LTA, so the observed responses must have been due to rapid LTA binding and stimulation of the cells. The two open columns at the far right depict results for controls that received LTA-free medium for two periods of 6 h each.

TABLE 2. Characterization of enterococcal LTA species containing two fatty acids[a]

Tested strain	Chain length (avg)[b]	Molar ratio to phosphorus of:		Glc_nGro fraction			
		AlaGro[c]	Glc_nGro[d]	GlcGro	Glc_2Gro	Glc_3Gro	Glc_4Gro
E. avium DSM 20679	29	0.26	0.16	0.88	0.12	—	—
E. casseliflavus DSM 20680	19	—	0.58	—	1.00	—	—
E. durans DSM 20633	28	0.38	0.38	0.35	0.65	—	—
E. faecalis DSM 20478	19	0.15	0.40	—	1.00	—	—
E. faecium DSM 2918	14	0.22	0.55	0.43	0.47	—	—
E. hirae ATCC 9790	21	—	0.69	0.56	0.24	0.04	0.17
E. hirae NCIB 8191	14	0.32	0.58	0.20	0.30	0.05	0.45
E. malodoratus DSM 20681	26	0.37	0.10	0.81	0.19	—	—

[a] Analyses were performed as described elsewhere (19, 30); —, not present.
[b] Molar ratio of glycerophosphate to glycolipid.
[c] Extent of glycerol (Gro) substitution with D-alanine ester.
[d] Extent of glycerol (Gro) substitution with glycosyl residues, which may be composed of mono-, di-, tri-, and tetra-α-D-glucopyranosyl residues (Glc_nGro, n = 1 to 4) connected with 1-2 interglycosidic linkages.

and chemical structure, including chain length, extent of glycosylation, and patterns of glycosyl substituents. If glycosyl residues play a role, and the stimulatory effect of the lipomannan of *M. luteus* supports this suggestion, differences in the fine structure, such as the order of glycosyl substituents along the chain, may be responsible for the divergent actions.

D-Alanine ester substituents seem not to affect the stimulatory effect of glycosylated LTAs, as was shown with *E. faecium* LTA before and after D-alanine ester was specifically removed.

In another set of experiments, we tested the possible effect of the number of fatty acids. On octyl-Sepharose, enterococcal LTAs separate into molecular species that have almost identical hydrophilic chains but contain two or four acyl residues (21, 23). Although these species can be predicted to differ in their critical micellar concentrations by several orders of magnitude, no significant difference in their action on monocytes was observed.

Synergism between LTA and pore-forming staphylococcal alpha-toxin. Previous work indicated that when monocytes are stimulated with LPS, first the IL-1β precursor accumulates intracellularly (1, 2, 27, 37a) and then the processing and release of IL-1β are accelerated through the action of pore-forming toxins (10). To discern whether there is a similar synergism between LTA and pore-forming toxins, we preincubated monocytes with stimulatory LTAs from enterococci for 1 h and then treated the monocytes with alpha-toxin from *S. aureus*. This protocol indeed led to a rapid appearance of IL-1β in the supernatants of toxin-treated cells (Fig. 4).

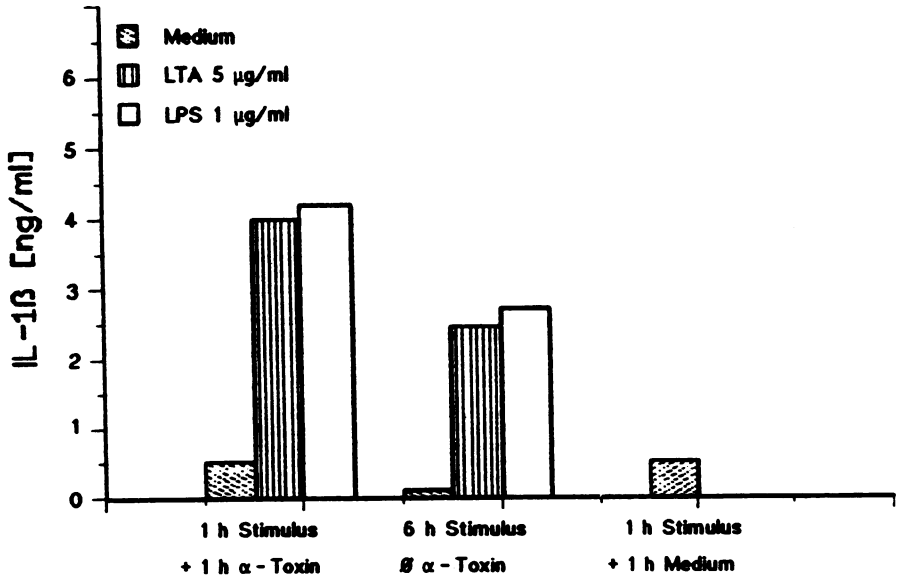

FIG. 4. Synergism between LTA and *S. aureus* alpha-toxin. (Left columns) Freshly isolated monocytes were prestimulated with LPS or LTA (from *E. hirae*, 5 µg/ml) for 1 h. Thereafter, the culture medium received 5 µg of *S. aureus* alpha-toxin per ml, and IL-1β was assayed in the supernatants after 1 h of incubation. The control received medium without LPS or LTA during the first incubation. (Center columns) Monocytes were stimulated for 6 h with LTA or LPS, without posttreatment with alpha-toxin. (Right column) Control in which cells were stimulated with LPS for 1 h and incubated for 1 h in medium without alpha-toxin. The pore-forming cytolysin induced the rapid release of IL-1β from prestimulated cells.

DISCUSSION

Our results indicate that certain LTAs can provoke maximal stimulation of IL-1β, IL-6, and TNF-α release from human monocytes, whereas LTAs derived from several medically important pathogens, including *S. aureus* and *S. pneumoniae*, are not strong inducers of cytokine production. We have been unable to identify common structural motifs that might explain the divergent stimulatory potentials of these molecules. Therefore, the behavior of any LTA cannot be predicted and, indeed, intraspecies variations may occur, as exemplified by the results obtained with enterococcal LTAs. The capacity of many enterococcal LTAs to induce the release of monokines is particularly interesting, since other factors of potential pathogenic relevance have not been identified. Our failure to detect a stimulatory effect of pneumococcal and staphylococcal LTAs on monocytes contrasts with the results from other investigations (32, 37). The reasons for these divergences are unknown.

The mode of binding of LTAs to cells and the events underlying the stimulation of monokine production require further study. Initial experiments conducted here indicate that binding requires fatty acid moieties and also may involve glycosyl residues and negatively charged, hydrophilic chains. Removal of fatty acids entirely abolishes all stimulatory effects, and the deacylated products are unable to block the action of whole LTAs. LTAs have been reported to bind to, and in some cases influence the function of, several cell types, including epithelial cells (3, 7, 13), platelets (4), polymorphonuclear leucocytes (15), lymphocytes (5), and erythrocytes (6), and binding to granulocytes and monocytes apparently stimulates the respiratory burst (26, 31). A requirement for fatty acids in mediating cell binding of LTA was previously shown (15, 41). In the present study, a potential role for the negatively charged poly(glycerophosphate) chains was additionally detected by the use of polycations. These agents selectively abrogated the stimulatory effects of LTAs but not of LPS, providing a unique means to exclude the possibility that the observed effects of LTAs were derived from contamination with LPS. In further support of this contention, several different preparations of any given LTA species always evoked the same positive or negative result in the stimulation assays. All LTA preparations were subject to very careful chemical analysis and fulfilled the most rigorous criteria for purity. 3-Hydroxytetradecanoic acid, a typical marker of LPS, was never detected.

The process of LTA binding could be temporarily distinguished and dissociated from that of cytokine production. A short incubation of cells with LTAs and replacement of the supernatant with LTA-free medium with or without poly-L-arginine revealed that the inhibitory effects of the polycation were confined to the prebinding period. We conclude that either negatively charged chains are directly involved in the binding process or polycations bind to the negatively charged groups on the surface of LTA micelles to form a shell which prevents the release of monomeric LTAs, which we believe represent the membrane-inserting species.

Monocyte stimulation was observed in the presence or absence of 10% autologous serum. Therefore, stimulation was not dependent on complement activation, which may be triggered by LTAs (18, 29, 34). Serum antibodies present in naturally occurring titers against LTAs were obviously not able to suppress the observed stimulatory effects.

Pore-forming bacterial toxins apparently act in synergy with LPS to induce the rapid release (within 60 min) of IL-1β from monocytes (10), and *S. aureus* alpha-toxin was similarly found to accelerate the release of IL-1β from cells pretreated with LTAs. This finding could have in vivo relevance during infections with bacteria that produce membrane-damaging toxins.

Certain LTAs share with LPS the potential of binding a 28-kDa protein of normal mouse serum which had originally been thought to be specific for the inner core region of LPS (11). The structural requirements of LTAs for this activity have not been identified. It is noteworthy that the ability of LTAs to bind the 28-kDa protein correlated with monocyte stimulatory capacity in some cases.

LTAs represent major membrane constituents of gram-positive bacteria (19, 40, 41), and these molecules may be shed from cells, particularly under the influence of β-lactam antibiotics (19, 41). Nevertheless, the role of LTAs in disease pathogenesis is obscure, and more recent studies have concentrated mainly on their possible function as adhesins and mediators of autoattack processes (3–7, 13–15, 35, 36, 38, 39). The present findings have revealed another, potentially important property of LTAs that merits attention.

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