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Macrophage Response to Bacteria: Induction of Marked Secretory and Cellular Activities by Lipoteichoic Acids

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Lipoteichoic acids (LTAs) from various bacterial species, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, and *Listeria monocytogenes*, were examined for the ability to induce secretory and cellular responses in a pure population of bone marrow-derived mononuclear phagocytes. Some of the highly purified LTAs, in particular LTAs from *Bacillus subtilis*, *S. pyogenes*, *E. faecalis*, and *Enterococcus hirae*, were able to affect each of the macrophage parameters measured, i.e., reductive capacity, secretion of tumor necrosis factor and nitrite, and tumoricidal activity. As after stimulation with whole organisms or other bacterial products, secretion of tumor necrosis factor induced by these LTAs reached its maximum within the first few hours of the interaction, while secretion of nitrite and tumoricidal activity required 24 to 36 h for full expression. Other purified LTAs, i.e., LTAs from *Streptococcus sanguis*, *S. pneumoniae*, and *L. monocytogenes*, as well as lipomannan from *Micrococcus luteus* affected only some of these parameters, while native LTA from *S. aureus* was inactive. There was no obvious correlation between biological activity and chain length, kind of glycosyl substituents, glycolipid structures, or fatty acid composition of LTAs. Deacylation of LTAs resulted in a complete loss of activity, and deacylated LTAs did not impair the activity of their acylated counterparts, suggesting that acyl chains may be essential for binding of LTA to the cell surface. The results demonstrate that some LTA species are potent inducers of macrophage secretory and cellular activities.

Macrophages are considered major effectors in the defense against bacterial infection (20, 42, 54). In vitro experiments with pure, lymphocyte-free bone marrow-derived mononuclear phagocytes that exhibit limited spontaneous functional activities have shown that these cells are able to respond to interaction with gram-negative and gram-positive bacteria with a marked enhancement of their functional activities (29, 30, 32, 34, 40). These findings indicated that macrophages do recognize these organisms in the absence of components of the specific immune system. However, the pattern of the macrophage response could vary considerably, depending on the type of bacteria. Analysis of the role of the structural constituents of bacteria in these processes has indicated that many effects of gram-negative bacteria on macrophages are mediated by bacterial lipopolysaccharide (LPS, endotoxin) and its biologically active lipid moiety, lipid A (18). LPS has been shown to trigger the release of tumor necrosis factor alpha (TNF-α [6]), interleukin-1 (IL-1 [23]), IL-6 (55), and nitrite (53) and to induce tumoricidal activity in macrophages (9). Bacterial peptidoglycans, major components of the cell wall of gram-positive bacteria (36), and their derivatives have been reported to mediate various immunomodulatory activities (52), to elicit inflammatory reactions (50), to exert an adjuvant effect on humoral and cell-mediated immune responses (1, 47), and to activate macrophages (19, 51). In this study, the secretory and cellular macrophage response to lipoteichoic acids (LTAs), including LTAs from clinically important pathogens, was examined.

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MATERIALS AND METHODS

Materials. Some LTAs (4, 5, 38) isolated in the laboratory of one of us (W.F.) and the succinylated lipomannan from *Micrococcus luteus* (14) have been characterized in the references given. These LTAs were extracted from mechanically disintegrated bacteria by hot phenol-water and purified from the crude extracts by hydrophobic interaction chromatography on octyl-Sepharose (15, 17). LTA species containing two or four fatty acids were collected separately. The preparations used all contained two fatty acids on the lipid anchor. LTA analyses were performed as described elsewhere (12, 38). Purified LTAs were shown to be free of detectable amounts of nucleic acid, protein, and polysaccharide contaminants (12, 14). Phospho- and glycolipids separated on extraction from LTA into the phenol layer (14), and residual traces would have been removed by hydrophobic interaction chromatography on octyl-Sepharose from which phospho- and glycolipids elute behind LTA (14a). For fatty acid analysis, LTAs were hydrolyzed (2 M HCl, 100°C, 2.5 h), and fatty acids were extracted with petroleum ether-chloroform (4:1, vol/vol), converted to methyl esters by using 10% methanolic BCl_3 (90°C, 10 min), and after partitioning of the mixture between water and chloroform, analyzed by gas-liquid chromatography (14). LTA was deacylated by mild alkaline treatment (0.2 M NaOH, 37°C, 60 min), neutralized with acetic acid, and used directly in the macrophage 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 (16), and the dealanylated LTA was converted to the sodium salt form and purified by hydrophobic interaction chromatography, using a centrifuge filtration device as previously described (17). The absence of deacylated LTA and lyso derivative (15) indicated that fatty acid esters had survived the mild alkaline treatment.

The initial experiments were performed with LTAs from *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mutans*, and *Streptococcus pyogenes*, purchased from Sigma, St. Louis, Mo. *Moraxella catarrhalis* and *Corynebacterium parvum* organisms were obtained, cultivated, and heat killed as previously described (29). Polymyxin B was from Sigma, and the Endospecy reagent set was from Seikagaku Kogyo Co. Ltd., Tokyo, Japan.

Search for LTA in *M. catarrhalis*. Disintegration of bacteria (0.1 g), extraction with hot phenol-water, and chromatography of the crude extract on octyl-Sepharose were performed as described for radiolabeled LTA (37). Small amounts of carbohydrate-containing material eluted with 40% propanol from octyl-Sepharose. The molar ratio of hexosyl equivalents to phosphorus of approximately 7:1 was reminiscent of LPS rather than LTA. The phenol layer, prepared as described previously (14), contained cardiolipin, phosphatidylethanolamine, and another phospholipid which chromatographed on two-dimensional thin-layer chromatography with phosphatidylcholine. Glycolipids and lower homologs of LTA (12) could not be detected.

LPS activity. To assess the possibility of LPS contamination, LPS activity of LTA preparations was determined by using the Endospecy reagent set. In addition, the ability of polymyxin B to interfere with NO secretion and tumoricidal activity induced in bone marrow-derived mononuclear phagocytes by the bacterial products was examined (28, 31).

BMMø. Bone marrow cells from femurs of male inbred DA rats were cultured in Iscove's modified Dulbecco medium conditioned with supernatant (final concentration, 10%) from strain L clone 929 cells (ATCC CCL1) as described previously (30, 34). On day 6, bone marrow-derived mononuclear phagocytes (BMMø) remaining adherent after repeated washing were cultured for the time interval indicated in medium alone (control) or in medium with one of the agents under examination before their functional activities were determined.

Assessment of macrophage functional activity. (i) Reductive capacity. BMMø were seeded in 96-well microplates (5×10^4 per well) and incubated for the time interval indicated in medium (control) or in medium supplemented with one of the agents under examination before cellular reductive capacity was determined in an MTT tetrazolium assay (44) by measuring the A_{570} in a Dynatech MR 700 microplate reader.

(ii) TNF-α activity. Supernatants harvested from 5×10^4 BMMø per well that had been cultured for various time intervals (usually 4 h) in medium supplemented with one of the agents were checked for cytolytic activity expressed against TNF-α-sensitive WEHI-164/13 cells in a 3-h MTT tetrazolium cytotoxicity test (35).

(iii) NO_2^- determination. One hundred microliters of cell-free supernatant harvested from 5×10^4 BMMø per well cultured for the time indicated (usually 24 h) in medium or in medium supplemented with one of the agents and/or sodium nitrite standard dilutions was mixed with 50 μl of Griess reagent (0.1% naphthylethylenediamine hydrochloride, 1% sulfonylamide, 3% H_3PO_4). After incubation for 10 min at room temperature, the nitrite concentration was determined by measuring the A_{550} in a Dynatech MR 700 microplate reader (35).

(iv) Assay of tumoricidal activity. Resting day 6 BMMø were incubated for 24 h in medium or in medium supplemented with one of the agents to be tested. The medium was then replaced by new medium supplemented with the same agent and prelabeled tumor targets, and tumoricidal activity was determined in a 36-h [^{14}C]thymidine release assay (35). Labeled P-815 murine mastocytoma cells and DA rat dimethylbenz(*a*)anthracene-induced (D-12 [30]) and methylcholanthrene-induced (M-1 and M-3 [30]) tumor cells were interacted for 36 h with BMMø (initial effector/target cell ratios of 2.5:1 and 5:1) before radioactivity in cell-free supernatants was measured, in order to calculate the percentage of specific isotope release (34). Tumoricidal activity of activated BMMø was expressed against all target cell lines but was particularly marked against P-815 mastocytoma cells; accordingly, only the results obtained with this tumor cell line are reported.

RESULTS

LTAs differ in the ability to trigger the macrophage activities. The initial studies were performed with commercially available LTAs. A comparative investigation showed that these compounds differed considerably in the ability to affect the functional activities of macrophages (Table 1). LTAs from *S. aureus* and *E. faecalis* were able to modulate each macrophage parameter examined. LTA from *B. subtilis*, being otherwise active, did not stimulate NO_2^- secretion significantly. LTA from *S. pyogenes* enhanced the secretion of TNF-α and nitrite but affected reductive capacity and tumoricidal activity only little; LTA from *S. mutans* was largely inactive (Table 1).

Analysis of the kinetics of the macrophage response to LTAs, represented for LTA from *E. faecalis* (Fig. 1) and *S. aureus* (Fig. 2), showed that the increase in secretion of TNF-α was an early event, reaching its maximum within the first few hours. On the other hand, NO_2^- secretion and expression of tumoricidal activity required a lag phase of 8 to 12 h until they began to evolve in parallel. From comparison of the kinetics of the macrophage activities triggered by LTAs from *E. faecalis* and *S. aureus*, it became clear that the macrophage response to biologically active LTAs could vary considerably. For example, incubation of BMMø with LTA from *E. faecalis* led to a continuous, marked increase in the secretion of NO_2^-; in contrast, tumoricidal activity reached a maximum after 24 h and then rapidly faded (Fig. 1). NO_2^- production and tumoricidal activity were also induced in parallel by LTA from *S. aureus*; however, after 24 h, the two parameters took a separate course: NO_2^- secretion ceased, whereas expression of tumoricidal activity was continuously increasing (Fig. 2).

Structural requirements for induction of the macrophage activities. In our attempt to arrive at a better understanding of the structural requirements for induction of the different macrophage activities, the ability of a series of pure, structurally defined LTAs to trigger the secretory and/or cellular responses in macrophages was also determined. The glycolipid and chain structures of these LTAs have been described previously (4, 5, 38). Because the fatty acid composition may play a role in binding to the cell membrane or receptors, fatty acid patterns were analyzed (Tables 2 and 3). As can be seen from Table 4, these LTAs also differed in the ability to stimulate macrophage activities. LTAs from *B. subtilis*, *S. pyogenes*, *E. faecalis* (native), and *Enterococcus hirae* enhanced each of the macrophage parameters studied. LTAs from *Streptococcus pneumoniae* and the lipomannan from *M. luteus* were unable to trigger tumoricidal activity, and LTA from *S. aureus* was completely inactive in its native alanylated form as well as after selective removal of alanine ester. As shown for LTA from *E. faecalis*, deacylation resulted in the complete loss of biological activity (Table

TABLE 1. Modulation of macrophage functional activity by commercially available LTAs[a]

Source of LTA	Concn (μg/ml)	Reductive capacity (%, 24 h)	Functional activity		Tumoricidal activity[b] (% thymidine release)
			TNF-α activity (% lysis)	NO_2^- secretion (μM/10^6 BMMø/24 h)	
None (control)		100	15–25	<1	0
B. subtilis	10	110–130[c]	30–60[c]	0–25	30–40[c]
S. aureus	10	100–120[c]			30–60[c]
	1		50–80[c]	60–80[c]	
				80–130[c]	30–60[c]
E. faecalis	1	80–90[c]			
	0.1		80–100[c]		
S. mutans	10	100–110	25–35	0–5	0–5
S. pyogenes	10	100		25–50[c]	0–15
	1		60–90[c]		

[a] Values are means or ranges from six to eight experiments, each performed in triplicate. LTAs were tested in concentrations of 10^{-2} to 10 μg/ml; the concentrations given were required to induce the changes indicated.
[b] Net isotope release from P-815 mastocytoma cells (initial effector/target cell ratio, 2.5:1).
[c] Statistically significantly different from control values ($P < 0.001$; Mann-Whitney U test).

4). This observation led to the question of whether the deacylated derivative might still be able to bind to and block specific binding sites. To assess this issue, BMMø were incubated for 2 h with deacylated LTA (5 μg/ml) before intact LTAs (1 to 10 μg/ml) were added to the culture. In no instance did incubation with deacylated LTA affect subsequent stimulation of any of the macrophage parameters by native LTAs (data not shown).

To assess the possibility that some of the effects of LTA were due to contaminating LPS, various tests were performed. First, 3-hydroxytetradecanoic acid, a marker of LPS, could not be detected when tested as previously described (13). Moreover, endotoxin determination in solutions of LTA from *B. subtilis* W23 and *S. pyogenes* II/D (each at 10 μg/ml) revealed a low endotoxin level comparable to that in medium alone. In addition, the cationic polypeptide polymyxin B (10 μg/ml), which markedly diminished NO_2^- secretion triggered in BMMø by LPS (28), affected NO_2^- secretion induced by these LTA preparations only little (data not shown). Finally, six of the eight purified LTA preparations tested were able to trigger in BMMø TNF-α-independent tumoricidal activity (Table 4); in contrast, LPS and lipid A were not able to induce such activity in this experimental system (28).

As previously reported, *M. catarrhalis* organisms differed from other gram-negative bacteria in their high potential to trigger tumoricidal macrophage activity (29). The causes for these differences are unknown. To ensure that *M. catarrhalis* organisms conformed with the doctrine that gram-negative bacteria are devoid of LTA, we attempted to isolate LTA from these bacteria. As described in Materials and Methods, this attempt took a negative course, indicating that

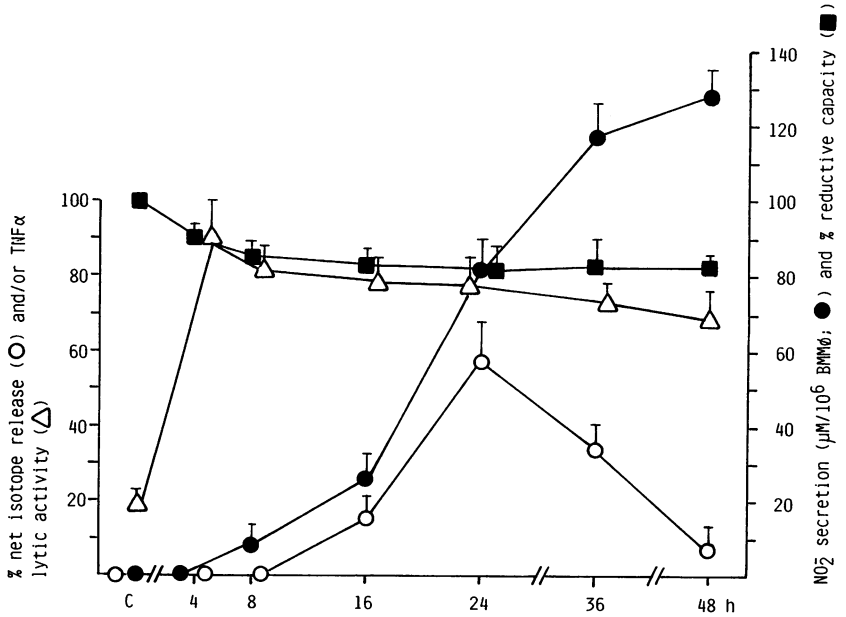

FIG. 1. Kinetics of macrophage activities induced by LTA from *E. faecalis* (1 μg/ml). Values are means and standard deviations from six experiments, each performed in triplicate. Tumoricidal activity was determined as net isotope release from P-815 mastocytoma cells at an initial effector/target cell ratio of 2.5:1.

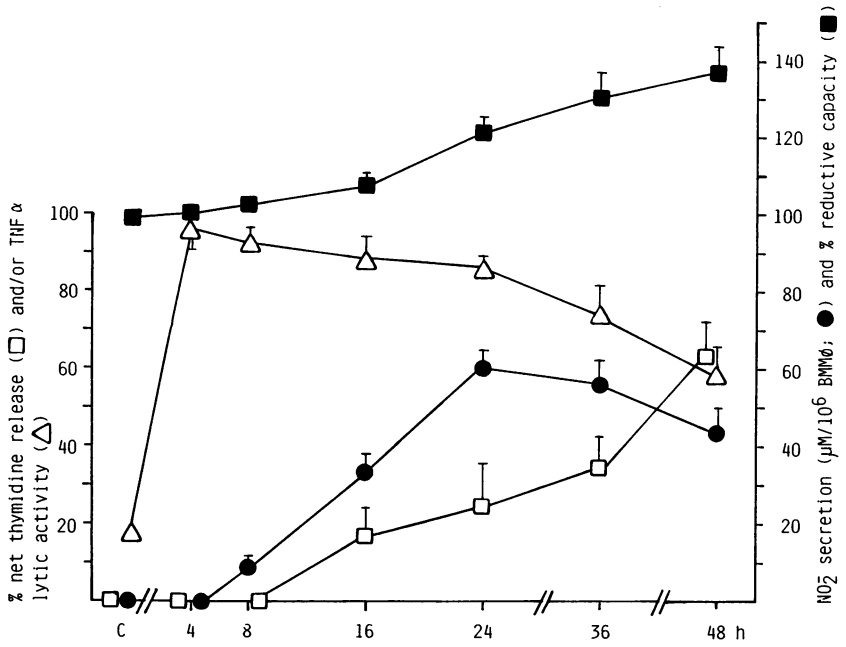

FIG. 2. Kinetics of macrophage activities triggered by LTA from *S. aureus* (1 μg/ml for stimulation of TNF-α and nitrite secretion and modulation of reductive capacity; 5 μg/ml for induction of tumoricidal activity). Values are means and standard deviations from five to eight experiments, each performed in triplicate. Tumoricidal activity was determined as for Fig. 1.

LTA did not contribute to the tumoricidal activity triggered by *M. catarrhalis* organisms (data not shown).

Macrophage activities differ in dependence on L-arginine. Earlier work with this experimental system has shown that production of NO and NO_2^- and expression of tumoricidal activity were abolished by analogs of L-arginine or arginase, indicating that these two functions depend on L-arginine (30, 32, 33, 35). We have now investigated the extent to which induction by LTAs of the various macrophage activities was affected by the presence of the L-arginine analog, N^G-monomethyl-L-arginine (NMMA). As shown for LTA from *S. aureus* and *E. faecalis* and for heat-killed *C. parvum* organisms, enhancement of the reductive capacity and secretion of TNF-α were in similar ranges regardless of the presence of NMMA (Table 5), suggesting that these two macrophage functions do not depend on L-arginine. In contrast, secretion of nitrite and expression of tumoricidal activity induced in macrophages by the same agents were both inhibited in a dose-dependent manner by NMMA (not shown) and were markedly suppressed by 10^{-4} M NMMA, thus confirming their dependence on L-arginine (Table 5).

DISCUSSION

LTAs, high-molecular-weight amphiphiles associated with the cell membrane of gram-positive bacteria, consisting of a hydrophobic glycolipid moiety and a hydrophilic chain which is either a 1,3-linked poly(glycerophosphate) or, in a limited number of bacteria, a poly(dihexosylglycerophosphate (11), have been considered surface-reactive antigens and/or agents which mediate attachment of certain bacteria to host tissues (3, 49). LTAs have been reported to induce nitric oxide synthase in vascular smooth muscle cells (2), to enhance carbon clearance (43), to have immunostimulatory and antineoplastic properties (21, 26, 48), to interact with classical (41) and alternative complement pathways (10, 25), to mediate (8, 58) or inhibit (59) lysis of cells, to bind to polymorphonuclear leukocytes (7), to stimulate the respiratory burst (39) and lysosomal enzyme release (22), and to induce tumoricidal activity in mononuclear phagocytes (21). Moreover, LTA from *S. pyogenes* suppressed the tumor growth of both solid- and ascites-type Meth A fibrosarcoma in mice (56). In a recent study, a number of chemically

TABLE 2. Straight-chain saturated and monounsaturated fatty acid composition of LTAs

Source of LTA	Content (mol%)[a]									Mean chain length
	12:0	14:0	16:1		16:0	18:1		18:0	19:cy	
			Δ7	Δ9		Δ9	Δ11			
S. pyogenes II/D	2.0	3.2	5.1	11.1	39.4	7.5	22.6	9.3	—	16.6
S. sanguis DSM 20567	—	6.2	8.7	3.4	40.1	3.2	26.3	12.2	—	16.7
S. pneumoniae R6	3.6	7.7	4.9	18.6	47.5	2.9	10.7	3.3	—	16.0
E. faecalis Kiel 27738	1.2	5.6	1.7	5.7	40.1	—	40.3	4.1	1.8	15.9
E. hirae ATCC 9790	—	3.2	2.2	3.8	40.0	4.3	38.8	3.5	4.2	16.9

[a] The first number represents the fatty acid chain length, and the second represents the number of double bonds; Δ*n* gives the location of the double bond; cy, cyclopropyl; —, not detectable.

TABLE 3. Saturated straight-chain and branched fatty acid composition of LTAs

Source of LTA	Content (mol %)[a]																	Mean chain length
	14:i	14:a	14:0	15:i	15:a	15:0	16:i	16:a	16:0	17:i	17:a	17:0	18:a	18:0	19:i	19:a	19:0	20:0	
B. subtilis W23	3.4	—	—	11.3	34.9	—	10.7	—	15.4	8.8	12.2	—	—	3.2	—	—	—	—	15.7
S. aureus DSM 20233	3.4	0.9	4.7	9.6	28.5	—	—	—	8.5	—	1.2	1.0	—	24.1	—	—	2.8	15.3	16.2
S. aureus[b]	—	2.8	2.4	6.7	31.1	—	—	—	9.1	3.1	7.9	—	—	17.5	2.4	4.0	—	13.0	16.7
L. monocytogenes NCTC 7973	—	—	1.6	9.9	43.6	1.1	0.7	1.2	4.7	1.9	28.2	0.8	3.3	2.7	—	—	—	—	14.8
M. luteus ATCC 4698	1.8	—	10.2	22.4	42.3	1.1	6.1	—	10.3	0.5	3.5	—	—	1.8	—	—	—	—	15.1

[a] *n*:a, anteiso branched; *n*:i, iso branched with the respective chain length (*n*); —, not detectable.
[b] Purchased; fraction retained on octyl-Sepharose (see text).

defined LTAs have been shown to stimulate in cultured human monocytes the secretion of TNF-α, IL-1β, and IL-6 (4).

In this study, five commercially available LTA preparations were first tested for the ability to induce in a pure population of BMMø the capacity to reduce MTT, the secretion of TNF-α and NO_2^-, and tumoricidal activity. The results show that these LTAs differed greatly in their potential to trigger these macrophage functions. LTAs from *E. faecalis* and *S. aureus* were active, and LTAs from *B. subtilis*, *S. pyogenes*, and *S. mutans* exhibited only limited activity (Tables 1 and 5). In an attempt to clarify the structural basis for the differences in biological activity, we performed additional experiments with another eight LTAs that were subjected to careful chemical analysis and fulfilled rigorous criteria for purity. With purified LTA preparations, various assays were performed to assess the possibility of LPS contamination. (i) Assays for 3-hydroxytetradecanoic acid, a marker of LPS (12), were negative. (ii) Endotoxin concentrations of LTA solutions were in a low range similar to that for tissue culture medium alone; (iii) polymyxin B, which efficiently suppressed induction of NO and NO_2^- in BMMø by LPS and lipid A (28), affected induction of NO and NO_2^- by LTA little if at all. (iv) LPS, lipid A, and LTA differed considerably in the ability to trigger TNFα-independent tumoricidal activity in this experimental system; LPS and lipid A exhibited no or only very limited activity, whereas the majority of LTA samples were active inducers of macrophage tumoricidal activity (Table 6). These findings strongly indicate that the described LTA effects were not due to contaminating LPS.

The data showing that some LTAs were able to modulate each of the four macrophage parameters examined (i.e., LTAs from *B. subtilis*, *S. pyogenes*, native *E. faecalis*, and *L. monocytogenes*) whereas other compounds affected only some functions (i.e., *S. sanguis* and *S. pneumoniae* LTAs and lipomannan) or were inactive (i.e., *S. aureus* LTA) (Table 4) confirmed that these molecules exhibit considerable divergence with respect to macrophage-activating potential. All stimulatory LTAs carried glycosyl substituents on their poly(glycerophosphate) chains. The unusual LTA of *S. pneumoniae* (12) and the lipomannan of *M. luteus* (14) were also carbohydrate rich. The enterococcal LTAs and LTA from *S. sanguis* contained α-D-glucopyranosyl residues

TABLE 4. Modulation of macrophage functional activity by highly purified LTAs and lipomannan[a]

LTA on lipomannan	Concn (μg/ml)	Reductive capacity (%, 24 h)	Functional activity		Tumoricidal activity[b] (% thymidine release)
			TNF-α activity (% lysis)	NO_2^- secretion (μM/10^6 BMMø/24 h)	
No LTA (control)		100	15–25	1	0
LTA from:					
B. subtilis W23	10	102–112[c]	60–80[c]	50–70[c]	30–40[c]
S. pyogenes II/D	10	105–115[c]	80–100[c]	40–50[c]	20–30[c]
S. sanguis DSM 20567	10	100		40–50[c]	20–30[c]
	1		60–80[c]	20–30[c]	10
S. pneumoniae R6	10	110–120[c]			
	1		60–80[c]		
E. faecalis Kiel 27738					
Native	10	105–115[c]	50–80[c]	5–15[c]	15–30[c]
Deacylated	10	100	15–25	0–3	0
E. hirae ATCC 9790	10	110–120[c]	50–80[c]	15–25[c]	15–25[c]
S. aureus DSM 20233					
Native	10	100–105	10–25	0	0
Dealanylated	10	100–105	5–15	0	0
L. monocytogenes NCTC 7973	10	100–105	50–80[c]	25–40[c]	20–35[c]
Lipomannan from *M. luteus* ATCC 4698	10	110–120[c]	60–90[c]	30–50[c]	10

[a] Values are means or ranges from five to eight experiments, each performed in triplicate. LTAs were tested in concentrations of 10^{-2} to 10 μg/ml. The concentrations given were required to induce the changes indicated.
[b] Net isotope release from P-815 mastocytoma cells (initial effector/target cell ratio, 2.5:1).
[c] Statistically significantly different from control values ($P < 0.001$; Mann-Whitney *U* test).

TABLE 5. Effects of NMMA on macrophage functional activities induced by LTAs and *C. parvum*[a]

Agent in culture medium	Reductive capacity (%, 24 h)	Functional activity		Tumoricidal activity[b] (% thymidine release)
		TNF-α activity (% lysis)	NO_2^- secretion (μM/10^6 BMMø/24 h)	
None (control)	100	15–25	0	0
S. aureus LTA (1 μg/ml)				
−NMMA	94 ± 5	75 ± 6	65 ± 8	65 ± 9
+NMMA (10^{-4} M)	88 ± 6	68 ± 7	17 ± 5[c]	0[c]
E. faecalis LTA (1 μg/ml)				
−NMMA	76 ± 4	86 ± 6	84 ± 9	61 ± 6
+NMMA (10^{-4} M)	72 ± 3	80 ± 9	9 ± 7[c]	0[c]
C. parvum (50 μg/ml)				
−NMMA	121 ± 6	71 ± 6	6 ± 2	64 ± 8
+NMMA (10^{-4} M)	120 ± 4	72 ± 8	7 ± 3	14 ± 5[c]

[a] Values are means ± standard deviations from six experiments, each performed in triplicate.
[b] Net isotope release from P-815 mastocytoma cells (initial effector/target cell ratio, 2.5:1).
[c] Statistically significantly different ($P < 0.001$; Mann-Whitney U test) from values for the corresponding experiment performed in the absence of NMMA.

and α-D-gluco-oligosaccharides, the LTA from *L. monocytogenes* contained α-D-galactopyranosyl residues, and the LTA from *B. subtilis* contained α-*N*-acetyl-D-glucosaminyl residues. The nonstimulatory LTA from *S. aureus* also contained α-*N*-acetylglucosaminyl residues, but no more than 5% of the glycerophosphate residues were substituted, compared with 25% in *B. subtilis* LTA. Considering the previously reported structures (4, 5, 38), there was no obvious relationship between the biological activities and chain lengths of LTAs, the nature of their glycosyl substituents, and their glycolipid structures. A recent study in which LTAs purified from the same bacterial strains as in this study were tested for the ability to induce in human monocytes the secretion of IL-1β, IL-6, and TNF-α led to a similar conclusion (4). It is noteworthy that with human monocytes, *S. aureus* LTA was also inactive while the other LTAs, except that of *S. pneumoniae*, displayed activities similar to those found in our experimental system. With both human monocytes and rat macrophages, deacylation of LTAs resulted in a complete loss of activity (4) (Table 4) and the deacylated LTAs did not impair the effects of their acylated counterparts. In both systems, therefore, the acyl chains appear to play an essential role in binding the LTAs to the cell surface (Table 4) (4, 7, 60). Despite this finding, a particular fatty acid composition is apparently not required, as LTAs possessing straight-chain saturated and unsaturated fatty acids (Table 2) may be as active as LTAs containing saturated straight-chain and branched fatty acids (Table 3). Whether the high content of octadecanoic and eicosanoic acids of *S. aureus* LTA contributes to inactivity requires further studies. The work with human monocytes further suggested that in addition to fatty acids, negatively charged groups of the chain might in some way be involved in the process of LTA binding (4). That each of the LTA preparations consistently triggered the same characteristic pattern of macrophage response suggests that, in keeping with earlier conclusions

TABLE 6. Modulation of functional activity of bone marrow-derived mononuclear phagocytes by bacteria and bacterial agents[a]

Type of bacteria or bacterial agent (reference)	Reductive capacity (%, 24 h)	Macrophage functional activity		Tumoricidal activity (% thymidine release)
		TNF-α activity (% lysis)	NO_2^- secretion (μM/10^6 BMMø/24 h)	
Gram-positive bacteria (29) (*E. faecalis*, *S. epidermidis*, *C. parvum*, *L. monocytogenes*)	110–160[b] (50–100 μg/ml)	40–80[b] (5–25 μg/ml)	2–60 (50–200 μg/ml)	>60[b] (1–25 μg/ml)
Gram-negative bacteria (29)				
E. coli, *P. aeruginosa*	110–130[b] (1–10 μg/ml)	30–70[b] (10–25 μg/ml)	50–80[b] (1–2.5 μg/ml)	50–80* (25–100 μg/ml)
M. catarrhalis	100–110 (1–2.5 μg/ml)	70–100[b] (0.5–1 μg/ml)	50–150[b] (1–2.5 μg/ml)	50–80[b] (1–2.5 μg/ml)
LPS (28) (*E. coli*, *K. pneumoniae*, *S. minnesota*)	80–120 (10^{-6}–10^{-3} μg/ml)	70–95[b] (10^{-6}–10^{-3} μg/ml)	60–100[b] (10^{-3} μg/ml)	<10 (10 μg/ml)
Peptidoglycan (31)				
Gram-positive bacteria (*B. subtilis*, *S. aureus*)		50–80[b] (1 μg/ml)	50–70[b] (10 μg/ml)	40–70[b] (1–5 μg/ml)
Gram-negative bacterium (*M. catarrhalis*)	100–130[b] (5 μg/ml)	70–100[b] (2.5–5 μg/ml)	50–80[b] (1 μg/ml)	40–60[b] (1–10 μg/ml)
Muramyl dipeptide	100 (10^{-8}–10^{-5} M)	50–80[b] (10^{-5} M)	5 (10^{-5} M)	0 (10^{-5} M)
LTA (*E. faecalis*, *B. subtilis*)	80–130 (1–10 μg/ml)	50–100[b] (0.1–1 μg/ml)	0–130 (1–10 μg/ml)	30–60[b] (1–10 μg/ml)

[a] Data are derived from references 28 to 31, 33, and this report. Values represent means or ranges from 5 to 12 experiments, each performed in triplicate. The concentrations given in parentheses were required to induce the functional changes indicated.
[b] Statistically significantly different from control values ($P < 0.001$; Mann-Whitney U test).

(28, 29, 33), induction of each of the macrophage activities examined in this study has distinctive structural requirements and that the processes of expression of these activities are not closely correlated. With human monocytes, no discrepancies in the production of the individual monokines were noted; i.e., production of TNF-α was always accompanied by production of IL-1β and IL-6 (4).

At present, we have no definite explanation for the divergent behavior of purchased and laboratory-prepared LTA from *S. aureus*. The fraction of the purchased sample that was retained on octyl-Sepharose (30% of total phosphorus) was similar in fatty acid composition to the laboratory-prepared sample (Table 3), had the same glycolipid structure, Glc(β1-6)Glc(β1-3)acyl$_2$Gro, and contained 20 glycerophosphate residues per chain, compared with 28 in the laboratory-prepared sample. In the purchased sample, 8 and 5% of the glycerophosphate residues were substituted with α- and β-*N*-acetyl-D-glucosaminyl residues, respectively, whereas in the laboratory-prepared sample, 5% were substituted with α-*N*-acetyl-D-glucosaminyl residues only (data not shown).

Earlier work showing that secretion of NO and NO_2^- and expression of tumoricidal activity by macrophages were both suppressed by agents that interfere with L-arginine, such as arginase or analogs of L-arginine, and that suppression was reversed by L-arginine provided evidence for the involvement of L-arginine-dependent mechanisms (24, 27, 29, 30, 33, 35). The present experiments with NMMA led to a similar conclusion (Table 5). However, induction and expression of the two functions did not always coincide. For example, LTAs from *S. pyogenes* (Table 1) and *S. pneumoniae* (Table 4) and lipomannan (Table 4) were active inducers of the secretion of NO_2^- but triggered only limited cytolytic activity; on the other hand, induction of tumoricidal activity by LTA from *E. faecalis* Kiel was associated with only little NO_2^- production (Table 4). Moreover, the kinetics of the two parameters did not always parallel each other (Fig. 1). The discrepancies in the macrophage (29, 30, 33) and vascular endothelial (46) systems regarding formation of nitric oxide and expression of biological activity suggested that nitric oxide may not by itself be the critical mediator. Therefore, one must consider the possibility that nitric oxide forms more stable complexes with thiols, iron(II), and/or protein, from which biologically active moieties could be released as required. Actually, various recent reports support such a concept (33, 45, 46, 57).

This work is the last in a series of studies in which the response of a pure, lymphocyte-free population of bone marrow-derived mononuclear phagocytes to heat-killed intact bacteria and to bacterial products was assessed (28–34). Comparison of the overall results (summarized in Table 6) makes clear that the various agents trigger a different macrophage response. Gram-positive organisms were potent in enhancing reduction of MTT, secretion of TNF-α, and tumoricidal activity, but most were poor in inducing the secretion of nitrite (29). Gram-negative organisms were active in stimulating reductive capacity and secretion of TNF-α and nitrite but were generally poor in triggering tumoricidal activity (29). *M. catarrhalis* organisms, which did not contain LTA in detectable amounts, differed from other gram-negative bacteria in that they proved to be highly potent inducers of tumoricidal activity (29). LPS and lipid A were active in modulating reduction of MTT and secretion of TNF-α and nitrite but were unable to trigger tumoricidal activity in our experimental system (28). Peptidoglycan from gram-positive and gram-negative bacteria enhanced each of the macrophage parameters studied and were especially efficient inducers of tumoricidal activity (31). It may therefore be argued that some of the tumoricidal activity triggered in macrophages by *M. catarrhalis* organisms might be mediated by peptidoglycan. As the amount of peptidoglycan in cell walls of gram-negative organisms is usually much lower than in gram-positive organisms (36), it is possible that other, still unidentified structural components of *M. catarrhalis* contribute to its striking macrophage-activating potential. Muramyl dipeptide, the water-soluble minimal structure of bacterial cell wall peptidoglycan required for adjuvancy, was not able to duplicate the pronounced effects of peptidoglycan. Pneumococcal polysaccharides and *Salmonella* oligosaccharides had only limited effects on macrophage functions and did not induce tumoricidal activity. This study now shows that in addition to LPS and peptidoglycan, LTAs from several gram-positive species exhibit an impressive macrophage-activating potential. As LTA is excreted from certain bacteria, in particular under the action of penicillin and other cell wall antibiotics (for references, see reference 11), it may well play a role in clinical infections.

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