**Synthetic Lipoteichoic Acid from *Staphylococcus aureus* Is a Potent Stimulus of Cytokine Release**

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**Abstract**

We recently purified lipoteichoic acid (LTA) from *Staphylococcus aureus* to more than 99% purity by a novel preparation method and deduced its structure with the first nuclear magnetic resonance (NMR) of a complete LTA. In contrast to Gram-negative lipopolysaccharides, this LTA requires the toll-like receptor (TLR)-2 and not TLR-4 for cytokine induction in monocytes and macrophages. To elucidate the structure–function relationships for LTA from *S. aureus*, the lipid anchor was prepared by either acidic hydrolysis of native LTA or chemical synthesis (gentiobiosyl-α-dimyristoylglycerol). Next, a complete LTA molecule with six glycerophosphate units carrying four alanine plus one N-acetyl-glucosamine substituent was synthesized, which displayed the same potency to activate monocytes as native LTA. However, 100–1,000 times higher concentrations of the lipid anchor were required for cytokine induction. It is worthy to note that replacing d-alanine with l-alanine blunted the effect indicating stereoselective recognition. The structure identification of this synthesized and biologically active LTA was proven by NMR and matrix-assisted laser desorption-ionization mass spectrometry. We concluded that the lipid anchor, with its fatty acids, represents an integral part of the immunostimulatory activity of LTA, but requires additional structural components on the polyglycerophosphate backbone.

**Key words:** tumor necrosis factor • Gram-positive bacteria • immunity, natural • structure–activity relationship • chemistry, organic

**Introduction**

The inflammatory responses to Gram-negative and Gram-positive bacteria can hardly be distinguished. However, although most of the immune stimulation caused by Gram-negative bacteria could be attributed to LPS as a general principle, no consensus as to its Gram-positive counterpart was reached during the last decades. LPS has been available in a pure, biologically active form since 1952 (1) and its active principle, the lipid anchor lipid A, was finally proven by chemical synthesis in 1985 (2). More than 45,000 Medline-listed scientific papers since 1966 describe biological activities of LPS.

Lipoteichoic acid (LTA) is found in most Gram-positive bacteria. Like LPS, it is an amphiphilic, negatively charged glycolipid. For three decades there has been intense discussion about whether or not LTA represents a counterpart to LPS. Many studies were performed with commercial preparations of poor quality and the significant activities reported could, in many instances, be traced back to LPS contaminations (3–6). When adequately purified, phenol-extracted LTA from both *Staphylococcus aureus* (7, 8) and *Enterococcus hirae* (9) turned out to be essentially inactive in inducing cytokine release as a measure of immunostimulatory activity. Furthermore, a synthetic LTA derivative was found to have no stimulatory activity (10–12). These observations have put into doubt the general role of LTA as an immunostimulator. We have recently shown that many of these concerns resulted from the inappropriate preparation methods used for LTA (13). After the phenol extraction, which is also used for LPS, we obtained partially degraded LTA. A novel, more gentle preparation scheme led to essentially homogenous, biologically active LTA. By means of structural analysis, we could attribute a crucial role to preserved d-alanine substituents of the polyglycerophosphate backbone of LTA (13). As a final proof of the biological activity of LTA, and to allow structure–function analysis, the chemical synthesis of a biologically ac-
tive LTA based on the structure of the one from *S. aureus*
was performed.

**Materials and Methods**

*Synthesis Strategy for LTA.* To confirm the structural assignment and the biological effect, compound 1, having six glycerol phosphate residues, was planned as the synthetic target. Thus, the proportion of the substituents α-Ala (66%), α-GlcNAc (17%), and hydrogen (17%) at the 2-O position of the glycerol moiety corresponds approximately to the distributions found in the natural product, i.e., α-Ala (70%), α-GlcNAc (15%), and hydrogen (17%), respectively (13). Compound 1 was assembled with the five building blocks shown in Fig. 1 based on a carefully selected protective group pattern. The phosphorous esters were obtained based on the phosphitamide methodology. The hydrolytically highly labile α-Ala residues were attached in the second to last step, which was immediately followed by deprotection (hydrogenolytic removal of all 21 benzyl groups) and purification by hydrophobic interaction chromatography on octyl Sepharose (13), thus providing compound 1 in good overall yield. The structure was confirmed by nuclear magnetic resonance (NMR) and mass spectrometry (MS) data (Fig. 2).

**LTA Structure Analysis.** NMR experiments were performed at 600.13 MHz (1H) and 300 K. The NMR spectra were related to 3-(trimethylsilyl) 3,3,2,2-tetradepsypropanionic acid Na salt (d4-TSPA). Homonuclear assignments were taken from double-quantum filtered correlation, total correlation, rotating frame Overhauser enhancement, and nuclear Overhauser effect spectra. 13C assignments were based on heteronuclear multiple-quantum correlation. Mass spectra were performed by gas chromatography-mass spectrometry (GC-MS) and matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) MS.

**Deacylation of Native LTA.** Hydrolysis of LTA from *S. aureus* (13) was performed at 37°C for 2 h with 0.1 M NaOH. Fatty acids of LTA were determined by GC-MS (Hewlett-Packard) as the respective methyl esters after methanolyis using 2 M HCl in methanol for 7 h at 85°C. The water phase was neutralized with NaHCO3 and freed of salts by dialysis in Spectrapor tubing with a molecular mass cut-off of 1,000 daltons.

![Figure 1. Scheme of the synthesis strategy for LTA analogue 1 from *S. aureus.*](image-url)
Native LTA Anchor Preparation. After hydrolyzing 220 mg LTA from *S. aureus* (13) with 5 ml hydrofluoric acid (48%) at 2°C for 42 h, 150 ml distilled water and 35 ml saturated NaHCO₃ were added. The lyophilisate was resuspended in 70 ml H₂O/CH₂Cl₂/MeOH (3:3:1) and centrifuged at 3,200 rpm for 7 min. The pellet was washed with CH₂Cl₂ and the water layer with CH₂Cl₂/MeOH (3:1). The collected organic phases were evaporated under vacuum at 40°C to a volume of ~15 ml and lyophilized. An aliquot of the lyophilisate (10 mg) was resolved in 200 µl methanol and subjected to a high performance thin layer chromatography plate silica gel 60 without fluorescent indicator using the solvent system CHCl₃/MeOH/H₂O (65:25:4). The Rₜ of the intact glycolipid was 0.68 (0.42 for anchor with one fatty acid). The substance line was scratched off and filtered over a glass filter (pore size 4) under vacuum after suspending with thin layer chromatography solvent. Finally, the solution was evaporated and lyophilized.

Murine Peritoneal Cell Preparation. Male C3H/HeN and C3H/HeJ mice were purchased from Charles River Laboratories. The animals received humane care in accordance with the National Institutes of Health guidelines and the legal requirements in Germany. Mice were put to sleep by terminal pentobarbital anesthesia (Narcorene™; Merial) and 10 ml of ice-cold PBS (GIBCO BRL) were injected into the peritoneal cavity. Animals were shaken gently and the lavage liquid was transferred to siliconized glass tubes (Vacutainer®; Becton Dickinson). After centrifugation, cells were resuspended in RPMI 1640 medium (BioWhittaker) containing 10% FCS (Boehringer) and 100 IU/ml penicillin/streptomycin (Biochrom), and transferred to 96-well cell culture plates (10⁵ cells/well). For the determination of cytokine induction by LPS, native LTA, or synthetic glycolipids, cells were stimulated immediately with pyrogen-free saline as solvent (Braun) at 37°C, 5% CO₂ in a humidified atmosphere overnight.

Cytokine Induction Assay. Cytokine release of human whole blood was determined as previously described (15) by incubating 800 µl isotonic sodium chloride solution, 200 µl human heparinized whole blood, and 10 µl stimuli. TNF-α was measured by sandwich ELISA (Endogen).

Results and Discussion

For LPS, the key role of fatty acids in binding to cellular receptors and the activation of monocyte/macrophages is...
finding that macrophages from LPS-nonresponsive C
LPS contamination. The latter notion is supported by the
nation by more than 1% and the LAL test largely excludes
combination of analytical methods excludes any contami-
LAL test does not detect this Gram-positive stimulus. The
lysate [LAL] Endochrome; Charles River Laboratories), ex-
were methylated. The most frequent fatty acids found were
fatty acids in the anchor (13) as ∼50% of the fatty acids
methylated. The most frequent fatty acids found were
tetradecanoic acids (≥36%), which were chosen for the
lipid anchor synthesis in the nonbranched form. The syn-
thetic molecule displayed activities similar to the natural
isolate (Fig. 3 A). The relative role of different fatty acids
will require further studies. Interestingly, when structurally
very similar compounds were tested, i.e., lactosyl disaccha-
rides that were ether-linked to fatty acids, we found a simi-
lar induction of cytokine release to the lipid anchor (un-
published data). Furthermore, when the racemic mixture of
both stereoisomers of the LTA anchor was compared with
the pure isomer, no major difference in cytokine induction
was observed (Fig. 3 A). In conclusion, the pattern recog-
nition of the lipid anchor is relatively loose, allowing a high
degree of variability of the anchor structure.

The fact that the lipid anchor alone was a factor of 100–
1,000 less potent in inducing cytokine release than native
LTA (Fig. 3 A), prompted the synthesis of a complete LTA
molecule. The synthesis strategy is depicted in Fig. 1. The
assembly of the five building blocks resulted in milligram
quantities of LTA, which was purified by hydrophobic in-
teraction chromatography. The identity of the molecule
was shown by NMR and MS (Fig. 2). The material was
negative in the Limulus test for Gram-negative endotoxin
(i.e., <10 pg LPS/mg LTA; Endosafe Limulus amebocyte
lysat [LAL] Endochrome; Charles River Laboratories), ex-
cluding an LPS contamination and demonstrating that the
LAL test does not detect this Gram-positive stimulus. The
combination of analytical methods excludes any contami-
nation by more than 1% and the LAL test largely excludes
LPS contamination. The latter notion is supported by the
finding that macrophages from LPS-nonresponsive C,H/
HeJ mice responded similarly to those of LPS-responsive
C,H/HeN mice to both the synthetic lipid anchor and na-
tive LTA (Fig. 4).

The resulting compound was as potent in inducing cy-
tokine formation in human whole blood as the native mol-
ecule (Fig. 3 B). We concluded that the structure of LTA
from S. aureus suggested (13), and indeed represents, an
immunostimulatory molecule. Although the natural com-
ounds termed LTA exist as a rather heterogeneous class of
apparently related molecules, the synthetic compounds ap-
pear to reflect key structural requirements for immune acti-
vation. We confirmed the key role of d-Ala substituents by
synthesizing the D-Ala derivative, which made the com-
ound less active by a factor of 100 (Fig. 3 B).

The biological activity of native LTA has been charac-

Figure 3. TNF-α induction by LTA anchor derivatives and synthetic
LTA. (A) The fission products of native LTA from S. aureus, intact an-
chor ( ), partially deacylated anchor ( ), and the synthetic compounds
( , pure diastereomer and , diastereomeric mixture) were used at the
concentrations indicated to induce TNF-α release in human whole blood
in comparison to native LTA from S. aureus (○). As a comparison, 10 ng/
mL LPS (Salmonella abortus equi) induced 7,620 ± 3,670 pg/ml TNF-α.
Data are given as means ± SEM of whole blood incubations of three do-
nors. (B) Human whole blood was incubated in the presence of synthetic
LTA carrying d-alanine ( ) or l-alanine ( ) substituents, or native LTA
(○) at the concentrations indicated.
terized recently in several studies (13, 15–19). The fact that the synthetic LTA behaved very similarly with regard to potency and pattern of cytokine induction as well as toll-like receptor use in vitro and in vivo (15, 16), suggests that it mirrors a major component of the biological isolate which, however, does not necessarily translate to all activities of the biological isolates.

Taken together, these studies show that LTA represents a Gram-positive endotoxin, i.e., a nonsecreted toxin of bacteria as originally defined. We suggest to broaden the use of the term endotoxin in this sense, to cover Gram-negative lipopolysaccharides, Gram-positive LTAs, and further immunostimulatory structures of the bacterial cell wall yet to be identified. Furthermore, we suggest to term the lipid anchor of LTA as “lipid B” in analogy to the name “lipid A” carried by the LPS anchor. Additional studies will be required to distinguish the different activities of LPS and LTA and define the effects of heterogeneities of the LTA molecule.

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